UNIVERSITY OF MICHIGAN
FEB 11 1952
MEDICAL LIBRARY

CANCER RESEARCH

VOL. 12 JANUARY 1952 No. 1

CONTENTS

S. Meryl Rose and Florence C. Rose. Tumor Agent Transformations in Amphibia 1

Henry Browning. The Action of Tumors on Normal Tissue Synchronously Transplanted into the Anterior Chamber of the Mouse Eye 13

Paulo R. F. Borges and Barbara J. Kvedar. A Mutation Producing Resistance to Several Transplantable Neoplasms in the C57 Black Strain of Mice 19

B. M. Peckham and R. R. Greene. Experimentally Produced Granulosa-Cell Tumors in Rats . 25

Hans L. Falk and Paul E. Steiner. The Identification of Aromatic Polycyclic Hydrocarbons in Carbon Blacks 30

Hans L. Falk and Paul E. Steiner. The Adsorption of 3,4-Benzpyrene and Pyrene by Carbon Blacks 40

Charles E. Wenner, Morris A. Spirtes, and Sidney Weinhouse. Metabolism of Neoplastic Tissue. II. A Survey of Enzymes of the Citric Acid Cycle in Transplanted Tumors 44

J. C. MacDonald, E. C. Miller, J. A. Miller, and H. P. Rusch. The Synergistic Action of Mixtures of Certain Hepatic Carcinogens 50

Paulo R. F. Borges and F. Duran-Reynals. On the Induction of Malignant Tumors in Pigeons by a Chicken Sarcoma Virus after Previous Adaptation of the Virus to Ducks 55

Maurice C. Smith, Paul A. Slattery, Michael B. Shimkin, Choh Hao Li, Raymond Lee, John C. Clarke, and William R. Lyons. The Effect of Pituitary Growth Hormone (Somatotrophin) on the Body Weight and Tumor Growth in C3H Mice Bearing a Transplantable Mammary Adenocarcinoma 59

Kingsley M. Stevens. Relation of Uptake of Radiophosphorus into Nucleic Acids to Age of Developing Chick Embryos 62

Anita H. Payne, Lola S. Kelly, and Margaret R. White. Effect of Neoplastic Tissue on the Turnover of Liver Nucleic Acids 65

Miriam S. Hoster, Quentin Van Winkle, Jacob G. Rabatin, and Herman A. Hoster. Macromolecular Particles Obtained from Human Neoplastic and Non-neoplastic Lymph Nodes. II. Electron Microscope Study 69

Announcement 75

THE OFFICIAL ORGAN OF THE
AMERICAN ASSOCIATION FOR CANCER RESEARCH, INC.
Published by THE UNIVERSITY OF CHICAGO PRESS

CANCER RESEARCH

This journal is sponsored by The American Association for Cancer Research, Inc.; The Anna Fuller Fund; The Jane Coffin Childs Memorial Fund for Medical Research; The Elsa U. Pardee Foundation; and The American Cancer Society.

HAROLD P. RUSCH, *Editor-in-Chief*
ELIZABETH B. POHLE, *Technical Editor*

Editorial Advisory Board

JOSEPH C. AUB
CARL A. BAUMANN
JOHN J. BITTNER
AUSTIN M. BRUES
DALE R. COMAN
E. V. COWDRY
HUGH J. CREECH
M. DEMEREC
F. DURAN-REYNALS
JACOB FURTH
W. U. GARDNER
SAMUEL GRAFF
HARRY S. N. GREENE
JESSE P. GREENSTEIN
ERICH HIRSCHBERG
CHARLES HUGGINS
ARTHUR KIRSCHBAUM
ALBERT L. LEHNINGER
C. C. LITTLE
BALDUIN LUCKÉ
G. BURROUGHS MIDER
JAMES A. MILLER
C. P. RHOADS
A. J. RIKER
MICHAEL B. SHIMKIN
PAUL E. STEINER
C. CHESTER STOCK
ROBERT E. STOWELL
ALBERT TANNENBAUM
SHIELDS WARREN
ARNOLD D. WELCH

CANCER RESEARCH is published monthly for Cancer Research, Inc., by the University of Chicago Press, 5750 Ellis Avenue, Chicago 37, Illinois. Subscriptions are by volume only and are payable in advance. The subscription price is $12.50 per volume; the price of single issues is $1.25. Orders for less than a full volume will be charged at the single-issue rate. Postage is prepaid by the publishers on all orders from the United States and its possessions. No extra charge is made for countries in the Pan American Postal Union. Postage is charged extra as follows: For Canada and Newfoundland, 50 cents per volume (total $13.00), 5 cents per issue (total $1.30); for all other countries in the Postal Union, $1.00 per volume (total $13.50), 10 cents per issue (total $1.35).

The following is an authorized agent:

For the British Empire, except North America and Australasia:

Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1, England. Prices of subscriptions and of single copies may be had on application.

Business communications, remittances (in United States currency or its equivalent), and subscriptions should be addressed to THE UNIVERSITY OF CHICAGO PRESS, 5750 Ellis Avenue, Chicago 37, Illinois. All other communications should be addressed to Harold P. Rusch, M.D., McArdle Memorial Laboratory, University of Wisconsin, Madison 6, Wisconsin.

Claims for missing numbers should be made within the month following the regular month of publication. The publishers expect to supply missing numbers free only when losses have been sustained in transit and when the reserve stock will permit.

No responsibility is accepted by the Editors, by Cancer Research, Inc., or by the publishers of *CANCER RESEARCH* for opinions expressed by contributors.

NOTICE TO SUBSCRIBERS: If you change your address, please notify us and your local postmaster immediately.

Entered as second-class matter, February 15, 1949, at the post office at Chicago, Ill., under the Act of March 3, 1879.

Acceptance for mailing at special rate of postage provided for in United States Postal Act of October 3, 1917, Section 1103, amended February 28, 1925, authorized June 1, 1950.

Copyright 1952 by Cancer Research, Inc.

Medical
Saxon

CANCER RESEARCH

VOLUME 12 JANUARY 1952 NUMBER 1

Tumor Agent Transformations in Amphibia*

S. MERYL ROSE AND FLORENCE C. ROSE

(Department of Zoology, University of Illinois, Urbana, Ill.)

INTRODUCTION

There is a reciprocal relationship between tumor agents and cellular differentiation. A particular agent may be introduced into only one type of differentiated or partially differentiated tissue. At the same time, an agent from a donor neoplasm will, when introduced into cells of a new host, not only induce rapid growth but also cause the host cells to become identical in appearance and in secretions to the donor cells. Some years ago Murphy (20) emphasized that these agents are concerned with cellular differentiation as well as with the initiation of abnormal growth. Murphy also pointed out the similarity of tumor agents to transformation agents of pneumococci. By 1914 the Rous group had discovered three different tumors from which the agent could be removed (24–27). When these tumor agents were injected into muscles, it was noted that certain cells (possibly connective tissue cells) changed to resemble those of the tumor furnishing the agent. For example, one of the tumors, a chondro-osteosarcoma, caused cells lying within the muscle to produce the peculiar cartilage and bone of the type found in the original tumor. A tumor agent may be passed through many generations and in each induce the same peculiar cell type. This indicates that something separable from cells can be added to other cells where it will be reproduced and at the same time direct the differentiation of the cells containing it.

Duran-Reynals demonstrated (10, 11) that tumor agents or viruses, as he called them, or microsomes, as they are called by Claude (7), from the Rous Sarcoma I of chickens could not only transform cells of ducks and other birds but that often during the reproduction of the particles in a foreign host the particles were themselves changed. The work of Duran-Reynals demonstrated that the chicken tumor virus was converted to a duck virus when introduced into the latter species. Furthermore, when new kinds of tumors arose in other tissues, a new agent with the ability to direct differentiation towards that of the new type of neoplastic tissue was obtained.

Frogs and salamanders offer peculiar advantages for the study of the ways in which tumor agents act during cellular differentiation and tumor formation. First, as will be shown below, tumor agents from frogs can become part of salamander cells. Secondly, during limb regeneration in salamanders there is the opportunity to learn both the effect of tumor agents on regenerating cells and the effect of the regeneration process on tumor cells. Thirdly, the cells of frogs and salamanders are sufficiently different so that one may in most cases distinguish them. This is invaluable when trying to learn whether one is dealing with a transformed cell or a transformed agent.

MATERIALS AND METHODS

PROPERTIES OF THE ORIGINAL TUMOR

The tumors used in this study belong to the class of Lucké renal carcinomas of *Rana pipiens*, from the Lake Champlain region (15, 17, 28). Approximately 2 per cent of the full grown mature frogs of northern Vermont and adjacent Quebec have renal tumors. Often, especially in tumors induced by tumor agent in the laboratory, there are several loci of origin in the kidney. Figure 1 shows a frog with five separate loci.

When carcinomas arise in the kidneys the renal tubule picture changes to a new and characteristic form. The change is one from a tubule with simple

* Supported by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

Received for publication June 26, 1951.

yk

epithelium to a solid cord several cells in diameter. The difference between normal tubules and tumor cords can be seen in Figure 2. As growth proceeds cords branch, and older cords may become cystic (Fig. 13) and be transformed to a more complicated pattern (Fig. 15). These photographs are of different tumors, but all stages from simple cords to complicated cysts may be found in different parts of one tumor. The growth pattern is a new and characteristic feature of this neoplastic tissue.

Specificity of Tumor Agent

Either the injection of glycerinated tumor or of dried tumor powder or the transplantation of living tumor to the anterior chamber of eyes may induce a typical renal carcinoma in the frog (16). Lucké and Schlumberger (17, 28) observed that the renal tumor agent invariably produces renal tumors of the same type and not other kinds of tumors. The specificity of the agent is such that after transfer to a new frog it lodges only in renal epithelium. There the combination of agent and cell produces the characteristic new type of tissue which continues to grow until the host dies.

Over the past 4 years, starting with natural tumors found in shipments of *Rana pipiens* from J. M. Hazen Co., Alburg, Vt., tumors have been routinely induced by transplanting bits of tumors, about 1 c.mm., into the anterior chamber of the eye. Approximately 30 per cent of the tumor implants grow in the anterior chamber after an initial period of regression lasting a week or longer. The implants which survive the initial period of regression grow as long as 5 months. During the growth period the anterior chamber may be filled by the tumor, and adjacent normal tissues may or may not be eroded away. The grafted tumor always regresses after its period of growth unless the host dies before the time for regression. In almost all cases (we have seen but one exception in 48 cases), a renal tumor which takes and grows in an eye chamber induces a tumor in the kidney of its host. In all but two cases the grafted tumor regressed at the time the induced host tumor began to grow. In these two cases graft and host tumor persisted together for a few weeks, and then the graft regressed and disappeared. The induced tumors which are almost certainly composed of host cells, plus the agent from the graft, as demonstrated by Briggs (5), do not regress. All have grown steadily until the host died. Presumably, as has been suggested before (21), the host can in time produce antibodies against the cells of other individuals but not against tumor agents once they have become a part of its own cells *in vivo*. Tumors are rarely induced in host kidneys by tumor implants unless the implant takes and grows.

Localization of the agent in kidney cells with subsequent production of the typical tumor and no other type of tumor is the rule when transplants are made within the *R. pipiens* from Alburg, Vt.

Lucké (16) has shown that the Vermont agent will not produce tumors in *Rana pipiens* of New Jersey, approximately 500 miles away. Recent work by Tweedell[1] has shown that intra-ocular Vermont renal tumor grafts do not induce renal tumors in the more distant *R. pipiens* of Wisconsin, Illinois, and Kentucky. Rather fine genetic differences, so fine that the frogs of Vermont and Wisconsin are distinguishable only with difficulty, are sufficient to prevent the co-operation of Wisconsin renal cells and Vermont agent in the production of a tumor cell.

These observations, along with those obtained on chickens by the Rous group and with those on mammary tumor agents in mice, indicate that a tumor agent when first obtained has very narrow specificity, growing only in a particular tissue of rather close genetic relatives. This was true of the Rous Sarcoma I agent before it had been passed through many chickens (24, 26). In the early days the agent would induce tumors in only very closely related chickens. Those of the same breed from the same farm were used in the first successful inductions by agent transfer. The tumor did not develop in birds of the same breed from other farms when they received the agent. Later, after transfers through many hosts, the agent changed and became much less narrow in its requirements for a host site. Some years later it would induce tumors in other breeds of chickens, and in ducks, guinea fowl, turkeys, and pheasants (12). As will be seen below there is great similarity between the changes in tumor agents of birds and amphibia.

Methods of Transplantation

Ordinarily, tissues of adult *Rana pipiens* and adult newts, *Triturus viridescens*, are incompatible. Any frog tissue, including the renal carcinoma, dies and is completely phagocytized within a few days. Two methods have been devised for its successful transplantation (23).

Small bits, usually measuring less than a cubic millimeter, were transplanted to the forearm into subcutaneous tunnels made by inserting the narrower blade of a pair of iridectomy scissors. An exit for release of pressure during the insertion of the graft was made at the proximal end of the subcutaneous tunnel in the elbow region. A piece of tumor, usually with a few grains of sulfadiazine powder, was inserted and made to lodge in the

[1] K. S. Tweedell, private communication.

middle of the tunnel away from the surface wounds. Forty to 50 per cent takes were obtained when both the host and the bits of graft were chilled in ice during the operation, and when the host with its graft was transferred immediately after the operation to a refrigerator at 6°–7° C. and kept there for 2 weeks except for 15-minute periods 2–4 times a day, when they were allowed to approach and sometimes reach 20° C.

Only two of 105 attempts to transplant the tumor were successful when the adult host and graft were not chilled during the operation, even though they were refrigerated after the operation in the usual way for the 2-week period. These results were checked histologically. Chill-grafted tumors can survive for at least 92 days after transplantation, including 14 days at 7° C. and 78 days at 20°–22° C. (Fig. 4). Like most of the tumors which survived for weeks, this one contained a fibrous coat secreted by the host. Two host fibroblasts with nuclei larger than the tumor nuclei can be seen in the upper left of Figure 4. Cells of newts are appreciably larger, tissue for tissue, than are cells of frogs.

An easier way to establish the renal carcinoma in Triturus is to protect it with pyribenzamine. If the newts are kept in a solution of 5×10^{-5} gm. of pyribenzamine/100 ml of 10 per cent Ringer's solution for 2 hours before receiving the graft and for a few days thereafter, the operation may be carried out at room temperature. The percentage of takes has been approximately 75. Figure 3 shows a 21-day graft in a forelimb of Triturus. The epithelial tissue is the frog renal carcinoma exhibiting typical morphological appearance. As in most of these grafts, there are dividing cells (one at right center of Fig. 3) and also phagocytes of the host. The larger nuclei of the host are easily identified.

No tumor cells could be found after 10 days in six newts which received transplants of the same tumor but which were not put in the pyribenzamine solution until immediately after receiving the tumor implant.

The short period just after transplantation is a critical one for the graft. Only if the tissues are cold or if pyribenzamine is already present at the time of transplantation do the grafts survive for even a few days. Once the initial critical period is over, the tumor graft can survive for at least a few weeks. Sometimes the volume of the tumor graft increases in the adult Triturus limb, but more often it remains the same for several weeks or slowly decreases. All have disappeared within 6 months. Hosts kept in pyribenzamine continuously do not retain their tumor grafts longer than hosts with successfully established grafts not kept in pyribenzamine. Apparently, the antihistamine protects against initial reaction but not against acquired immunity.

RESULTS

Tumors Induced in Triturus

The results are divided into three groups: effects of grafts on adult newts, on the eft stage, and on regenerating tissues of both eft and adult.

Grafts to adult Triturus.—Four hundred and sixty full-grown adult Triturus received subcutaneous grafts in the forearm. Twenty received intra-ocular implants, and 100 more received intra-abdominal injections of the anuran renal carcinomas. In all cases there was no sign of grafted tumor a few months later, and in none, including 90 which were kept under observation for a year, were tumors induced. Furthermore, as background for the results to follow, no natural tumors were found in any of the several thousand Triturus which were kept in our laboratory. Tumors in salamanders may be quite rare, and no natural tumors have been reported for the genus Triturus in the Schlumberger and Lucké catalogue (28).

There was a different result in some cases either when the bits of tumor were grafted to adult limbs which were subsequently amputated through the tumor implant to test for regenerative ability or when the grafts were made to limbs of young efts.

Grafts in regenerating limbs of adult Triturus.—A tumor graft (Fig. 5) may be seen lying in an adult Triturus forelimb between skin on the right and muscle on the left. The anuran tumor tissue is easily distinguishable because of the smaller nuclei, prominent nucleoli, differences in staining reaction in hematoxylin-eosin-Azure II preparations and because of its typical cords. A section of the tumor used as the source is shown in Figure 13. Figure 5 is a longitudinal section through the amputated section of the forearm extending distally from the amputation surface. This tumor implant, unlike most, had grown appreciably and at the time of amputation, 20 days after being chill-grafted, extended all the way from wrist to elbow. A large portion of the tumor lay proximal to the level of amputation in the stump.

Grafted tumor lying in the region of regeneration blastema formation loses its typical morphological appearance, just as do all the normal tissues near an amputation surface during the formation of a regenerating blastema. In the particular limb under consideration, a blastema had formed, just as in 27 other limbs which received tumor implants at the same time. But in this one limb, after the blastema had formed at the normal time and the tumor as such had disappeared, neither growth

nor differentiation were externally visible. This unusual behavior prompted its removal and histological study. In Figure 7 the persistent blastema is shown in median longitudinal section. It is normal in appearance, showing, at the top of the photograph, the epidermal wound cover and beneath it many mesenchymal type regeneration cells. At the base, new cartilage was forming. The unusual feature is that an undifferentiated blastema should persist so long. Other regenerating limbs of the same series had reached well differentiated finger-bud stages. It is to be noted that cartilage had begun to differentiate in the persistent blastema. Ten days after amputation of the blastema, the first Triturus tumor we had ever seen was apparent as a protruding mound in the other forelimb—a limb which had not been operated upon. The tumor was composed of abnormally arranged chondrocytes and cartilaginous matrix continuous with normal cartilage of the distal head of the humerus (Fig. 6). The chondrosarcoma lay above the distal end of the humerus in the photograph. It arose in the same region of the untreated limb as that in which regenerating cartilage and tumor graft had lain in the treated limb. The question arose whether the same tumor agent, formerly so limited in its affinity, affecting only renal epithelium of *Rana pipiens*, could be recovered from the chondrosarcoma of the salamander. First, however, four small pieces of the tumor were subcultured for 6 weeks in limbs of other adult Triturus. Two of the subcultures regressed, and two maintained themselves but did not grow, as is the case when amphibian tumors are transplanted without protection to other individuals in regions other than eye chambers. One piece, less than a cubic millimeter in volume, was minced and then blended for 5 minutes in 50 ml. of Ringer's solution at 20°–23° C. One ml. of the dilute suspension was injected into coeloms of twenty *Rana pipiens* of Vermont. Of these, ten survived for several months. One of the ten developed a tumor which was first observed 46 days after the injection. It arose as a protuberance at the distal end of the right tibia. As growth continued, the right tibia enlarged and became irregular along its entire length until it was about 25 mm. in diameter in the broadest region at the distal end. At the peak of the growth of the tumor the right femur was also slightly enlarged and irregular. In a few weeks blood vessels leading to the foot were partially blocked. At this time the limb with the larger part of the tumor was amputated below the knee. The excess growth involved cartilage, bone, and a marrow-like tissue, as seen in Figure 8. Forty-nine days after the removal of most of the tumor, a skeletal growth arose at the base of the proximal phalanx of the fourth toe of the left foot. The animal finally died with a renal tumor 5 months after receiving the salamander chondrosarcoma, when almost all the kidney had been replaced by a typical renal carcinoma. The skeletal growths had almost completely regressed by the time of death.

Two types of transfer were made from the original skeletal growth of the frog at the time of amputation. In the first type of transfer, small pieces of the tibial growth were implanted into the anterior chamber of the eye of three adult *Rana pipiens* from Vermont. The grafts took and grew in the eyes of the hosts after a latent period of 2–4 months. Normal cartilage from the same limb was also transplanted to chambers of the eye where it persisted for several months but did not grow. A photograph of one of the growing skeletal tumor implants shows a triangular osteogenic tumor lying in the anterior chamber of a host eye (Fig. 16). Such grafts are well supplied by blood vessels running in strands of pigmented tissue from the iris. In this first generation of transfer, the non-osseous components, cartilage and marrow, disappeared, and only bone grew appreciably. The one shown in the Figure, like the others which grew, increased to several times its original diameter at the time of transplantation. All three of the animals within whose eye chambers the osteogenic tumor grew developed renal tumors of their own within 2 months of the time the implant in the eye began to grow. A section of one of them is shown (Fig. 15). These renal carcinomas appearing in host kidneys were typical in appearance and in growth rate and killed their hosts in 3–5 months. None of twenty control frogs which had not received any transplants developed renal carcinomas within the period of the experiment. All three of the animals in which osteogenic tumor grafts were successful developed renal carcinomas. Apparently the agent inducing the typical renal tumors was coming from an osteogenic tumor.

All nonautoplastic transplants, whether of normal tissue which does not grow in eye chambers, or neoplastic tissue which may grow, finally regress. The time for the beginning of regression of the intra-ocular osteogenic tumor grafts was from 3 to 7 months. Before one of them had completely regressed, it was divided into ten parts and re-implanted into ten more eye chambers. In only one case did the second generation implant take and grow, and the host in which it did was the only one of the ten in which a renal tumor developed within 2 months. It seems to be a general rule both for small renal and for small osteogenic tumor implants in eyes that only those implants which grow induce

tumors in host kidneys. It was strongly suspected after these data had been collected that an agent which will induce a typical renal carcinoma had passed from a renal carcinoma of a frog to a chondrosarcoma of a salamander to a chondro-osteogenic sarcoma of a frog and had finally returned to renal epithelium of a frog where it induced the typical carcinoma (Chart 1). More conclusive evidence that an agent can be part of tumor cells of a different tissue of a different species and yet retain enough of its original nature to induce the original type of tumor comes from an experiment in which transfer was made to larval salamanders, but this will be described after we have followed other transfers of the original osteogenic tumor.

A 5-c.mm. piece of the same osteogenic tumor which had provided the tissue transferred as intraocular implants was blended for 5 minutes in 200 ml. of Ringer's solution, and 0.4–0.6 ml. of the suspension was injected into the abdominal cavities of 23 *Rana pipiens* of Vermont. Five of these showed localized skeletal growths within 23 days of the intra-abdominal injection. The preparation was from a tumor at the distal end of a right tibia. The induced tumors appeared at the distal end of a left tibia in one frog, and in three other frogs primary tumors appeared at a locus in one limb and were followed by secondary tumors in the same locus of the contralateral limb. In one case the tumors arose at the joint between the third and fourth phalanges of the fourth toe of both feet. The one on the right foot extended 6 mm. beyond the surface of the toe and the one on the left foot 3 mm. In a second frog the growths appeared between the first and second phalanges of the third finger of both hands and were approximately 5 mm. in diameter. A third had both its metatarsal regions enlarged to approximately double the normal size. All three of these animals had no other tumors.

All the induced skeletal tumors of this generation, like the two of the former generation, were temporary growths and regressed within a few months. They arose more rapidly and grew more rapidly, but the affected cells died in time.

It had seemed a strange coincidence when a chondrosarcoma had appeared at the right elbow of a newt shortly after regenerating cartilage and renal tumor had been together at the left elbow. Now, with these three cases of tumors appearing in bilaterally identical regions, it no longer seemed possible that we were dealing with simple coincidence. It appeared more likely that, after a tumor agent had entered cells on one side of the body, a new type of tumor agent might emerge. Part of the affinity of the new agent seemed to have been conferred upon it by the host cell, because it then was most likely to cause growth in similar cells on the other side of the body. Each of the tumors of the last generation was blended 10 days after it appeared, and such preparations of each were injected into abdominal cavities of adult *Rana pipiens*. The concentration was higher this time—0.2-ml. injections of approximately 1 part tumor to 100 of Ringer's solution. Of 80 frogs receiving injections of the various skeletal tumors, 45 died within 2 weeks. All but 2 of these exhibited localized histolysis and hemorrhagic areas. Many tissues in different frogs, but muscle more than others, seemed to be affected. All the tumors used as donors gave the same results.

There was little evidence of affinity for skeletal tissue. There were four cases of localized temporary hypertrophy of skeleton, all in legs, but many tissues and organs all over the body were affected. In the skeletal growths hypertrophy began within 3 days of the time of injection of blended skeletal tumor. All growths were temporary, lasting from 3 months to 10 months. Some of the tumors, particularly one which arose in an oviduct, were invasive sarcomas. Two others which began in mesenteries or peritoneum near the site of injection spread as sheets of neoplastic tissue over viscera and invaded them at points. A nodule in one such sheet is seen in Figure 18. In the same frog, liver, uterus, intestine, and kidney had been invaded. Partially disorganized kidney through which tumor had spread is shown in Figure 19. Leg muscles of another frog were involved. Sections revealed anaplastic tissue composed of cells with large pale nuclei and large nucleoli. Wherever this tissue was present there was marked sarcolysis. On the left in Figure 20 are two muscle fibers, disrupted and partially dissolved. It appeared either that the agents had lost their affinity for a particular tissue or that no specificity could be recognized when higher concentrations cause such widespread hypertrophy and histolysis. In the early days of this work bacteria were suspected of being the cause of the histolytic lesions. But that bacteria are not the causal agents of all or even most of the lesions follows from several facts which will be discussed after further presentation of data. The multiple effects of the agent modified by passage between frog and salamander and subsequently through frogs are similar to those reported by Duran-Reynals after modification of Rous Sarcoma I agent during transfer between chickens and other birds.

Instead of a latent period of 2–3 months between the introduction of the tumor and the induc-

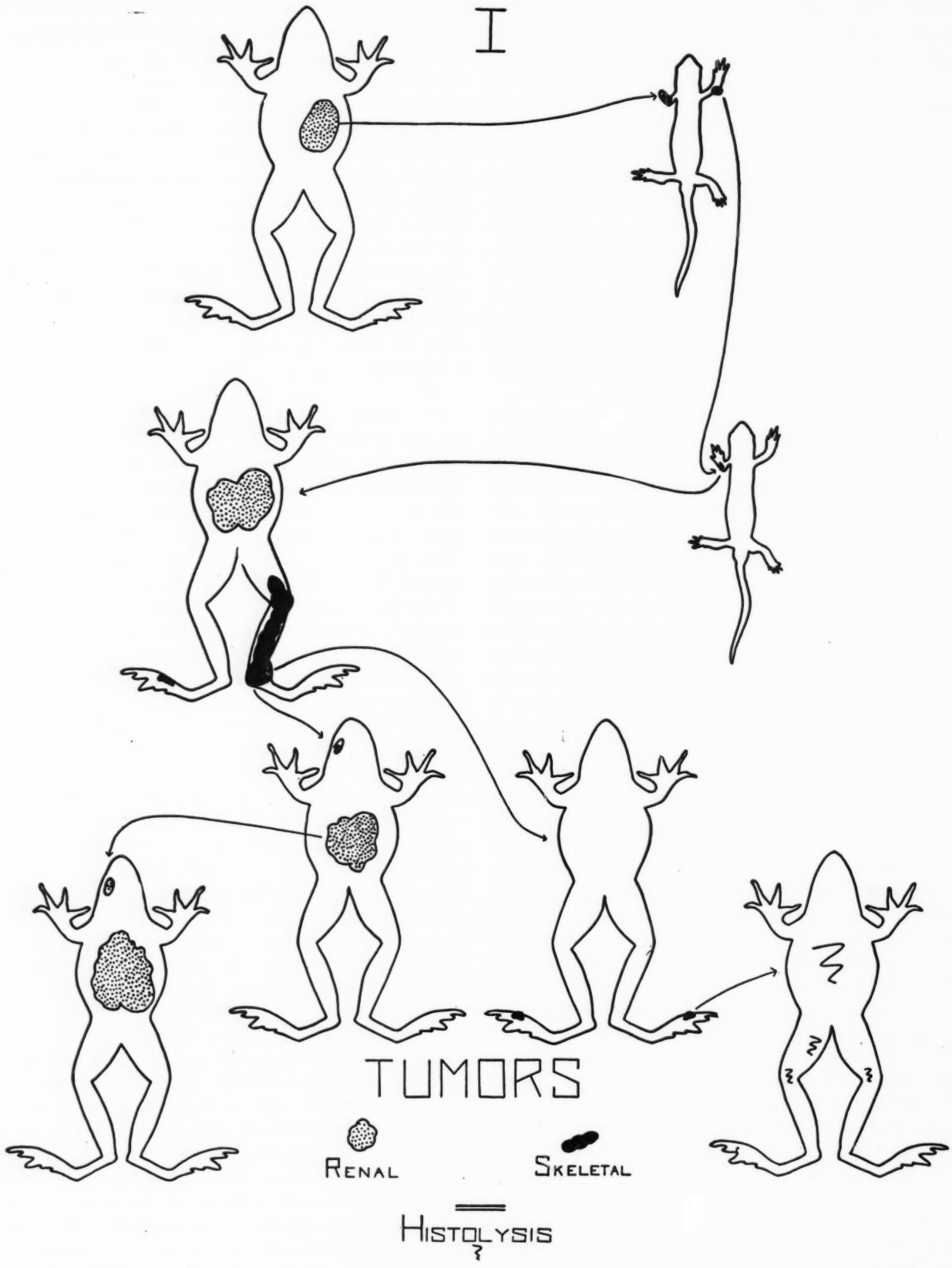


CHART 1.—Summary of tumors induced after transfer of a frog renal tumor agent to regenerating cartilage of an adult salamander.

tion of new ones, as had always occurred when an unmodified renal agent was used, hypertrophy began in some cases within a few days. Cells were more abnormal, growth was more rapid, and many of the cells seemed to lyse just behind the growing front. In all cases, unless the affected animal died within a few weeks, the induced tumors regressed completely. The cells of the typical renal carcinoma are more normal in appearance, grow more slowly, and never regress in the animal of origin. The agents used in this last generation of transfers induced rapid growth, but in all cases growth was followed by death of the part which had grown. In five animals marked localized histolysis was noted without previous hypertrophy. The places in which this occurred were bone of head, skeletal muscle of leg, and abdominal body wall near the site of injection.

Grafts to young Triturus.—Pieces of another anuran renal carcinoma (Fig. 14) were transplanted to forelimbs of eleven young efts not more than 2 months beyond the larval stage. No special precautions were taken to protect the grafts, except that hosts were kept at 7° C. for 1 day after the operation. Tumor grafts seem to have taken in eight of the eleven efts. Uncertainty arises from the fact that, although cells grow and spread from the region of implantation, they no longer have the appearance or the tissue form of a Lucké carcinoma. These growths, presumably of frog cells, were readily transplanted to other efts but failed to grow in 40 adult Triturus. In one, the growth was established in the anterior chamber of a frog's eye chamber and induced a renal carcinoma.

Anaplastic tumors spread from the site of implantation in the forearm. The route was mainly subcutaneous, marked by spread of anaplasia, formation of pearls of viscous fluid surrounded by fibrous capsules. Occasionally tongues of tumor tissue deviated from the subcutaneous route and progressed through the muscle. Occasionally epidermal ulcers appeared above the spreading tumor. The tumor tissue, which in one case had spread to the cervical region, can be seen running almost vertically through the center of Figure 9. It is flanked on the left by muscle and on the right by skin. Another which had reached the head in a few weeks can be seen in the skin of the head (Fig. 10). At the left lie skin glands ordinarily surrounded by dermis but which in this case had been replaced by anaplastic tissue. To the right is hypertrophied Triturus epidermis which had begun to ulcerate. Limbs of efts, like those of adults, regenerate even when tumor is present at the level of amputation. Tumor disappears during the reorganization, and in 20 of 23 cases regeneration was normal and the regenerates were maintained. In the other three cases, after amputation the limb stumps had passed through the stages of loss of distal organization, blastema formation, morphogenesis, and growth, only to be followed by partial or complete resorption of the regenerate. One was fixed at a late stage in the process of reorganization (Fig. 11). The figure shows a section of a regenerant which was beginning to suffer histolysis. The central region in Figure 11 is an area of destruction of recently regenerated tissue. A front of disruption of regenerated cartilage may be seen on the right. Epidermis to the left is hypertrophied, and much of it is necrotic.

One cannot decide until further tests are made whether the agent was transferred to Triturus cells during regeneration of eft limbs. However, in these young salamanders, in contrast to the findings in adults, tumors arose in host tissues distant from the site of implantation. In three efts tumors composed of host cells appeared in the wake of the spreading neoplasia. One was a myxosarcoma arising in an axilla, and two were chondrosarcomas, one arising from a scapula and the other from a humerus (Fig. 12).

The chondrosarcoma from the upper arm was used as a source of modified agent for a retest of its ability to induce renal carcinomas in frogs.

It may be of interest in passing to note that, upon amputation through the middle of it, the chondrosarcoma completely disappeared from the limb stump and that all tissues including cartilage regenerated normally. A section of the regenerated elbow is shown in Figure 17.

Small bits of chondrosarcoma were removed from the amputated limb and transferred without blending or grinding to anterior chambers of the eye of *R. pipiens* of both Vermont and Wisconsin. Whole cells were used in this experiment, because it was feared that, when excessive amounts of agent were released from disrupted cells in the former experiment, specificity might have been masked by more general effects.

Typical renal carcinomas were induced in all the hosts, both from Vermont and from Wisconsin, in which the chondrosarcoma had been established. Only the Wisconsin series will be considered further.

It is significant that renal carcinomas appeared in the only three Wisconsin frogs in which this second Triturus chondrosarcoma had been established. There had been 80 failures and no successes in trying to induce renal carcinomas in Wisconsin hosts by grafting Vermont renal carcinomas to eye chambers (28). Furthermore, although renal carcinomas very much like the Lucké renal carcino-

mas of Vermont frogs do occur in *Rana pipiens* of Wisconsin, they are relatively rare. We have found only two in more than 2,000 frogs examined. None of several hundred Wisconsin frogs has developed a spontaneous renal tumor in our laboratory. Another fact which greatly increases the probability that the recovered renal carcinomas were induced by an agent from the chondrosarcoma is that the Wisconsin hosts were only half-grown. We have never found spontaneous tumors in frogs so small. Altogether, the possibility that these tumors were not induced is exceedingly small.

In this series, unlike the former, only renal tumors arose in frogs after the introduction of the agent from a salamander chondrosarcoma. However, one of the renal carcinomas induced in a Wisconsin *R. pipiens* has been used as a donor of eye implants to *R. pipiens* of Wisconsin, Illinois, and Minnesota, and in all groups skeletal tumors have been induced. Of fifteen Minnesota frogs which had received an anterior chamber implant, one developed a skeletal tumor; of ten from Illinois, two developed skeletal tumors; and two developed skeletal tumors out of a group of fifteen from Wisconsin. One of these two Wisconsin frogs has now developed a renal tumor two months after the intraocular implantation. The usual time for renal tumor induction is 2–3 months, and more may appear later. One of the Wisconsin frogs had skeletal growths starting at both knees and in the right wrist. The right wrist tumor has been used as a source of tumor for implantation in eye chambers of other Wisconsin frogs. This skeletal growth, as did the one in the other series, caused hypertrophy and histolysis within a few days after implantation. In the former series the tumor was blended before injection, and in this series, in order to escape immediate large doses of agent, bits of intact tumor were implanted in eye chambers. More died in the former series, but widespread effects on a variety of tissues were noted after both methods of transfer. Three of ten Wisconsin frogs died within 15 days after implantation after localized hypertrophy, beginning at joints and spreading along long bones, was followed by hemorrhages in the same sites. Two of the frogs died from reactions beginning in phalanges and metatarsals, and the other which reacted to the wrist tumor implant died within 15 days of histolyzing growths of the two knees, the left wrist, and left metatarsal region. A fourth frog died in 20 days with hemorrhagic lesions without having shown any previous hypertrophy. The lesions, as is fairly common, were bilateral and, although not confined to the sartorius muscle, were predominantly located there (Fig. 23). This series is summarized in Chart 2.

Quite similar transformations were noted in both series of agent transfers. In the first, the Lucké agent was first changed in regenerating salamander chondroblasts, acquiring an affinity for skeletal tissues while retaining its ability to grow in and induce tumors in renal epithelium. In the second series the same transformation of the agent was observed, but this time the original transformation occurred in chondroblasts or chondrocytes of a young salamander. In both series agents after growth in foreign tissue were noted more for their ability to cause cells harboring them to lyse than for their ability to cause persistent growth of the host cells. The resemblance of some of these lesions to bacterial lesions is striking. However, the lesions in this series in which tumor was implanted arose only in those hosts in which the tumor took and grew. If bacteria were the causal agents, the lesions should appear, at least occasionally, after any transplantation, because bacteria are probably transplanted every time. The fact that the lesions in question did not appear at random after transplantation but only following growth of implanted tumor demonstrates that the causal agent is associated with the growing tumor implant and not with bacteria which might enter the host with every transplantation. Furthermore, the histolytic type of lesion did not follow implantation of all types of tumors. This type of lesion never followed implantation of an original unchanged tumor. Only when the agent had been a part of at least two tissues, and usually three, did it induce the histolytic type of lesion. This happened fairly regularly in three different series.

Some of the lesions in question were those which appeared exactly contralateral to induced new types of growth. It is difficult to understand how this phenomenon, which occurred 6 times and in about 10 per cent of the cases, could be accounted for by a bacterial hypothesis.

Transformation during Transplantation between Geographical Races

There is very little response on the part of tissues surrounding an eye chamber to an implant of a renal tumor when the cells of the tumor are from an animal of the same geographical region. The reaction of eyes of frogs from either Vermont or Wisconsin to a renal tumor from a frog of the other region is much greater, and very rarely can the tumor of one race be grafted successfully into the other race. This reaction between the relatively closely related frogs of Vermont and Wisconsin is much greater than the reaction between more distantly related frogs (28). Eye chambers become

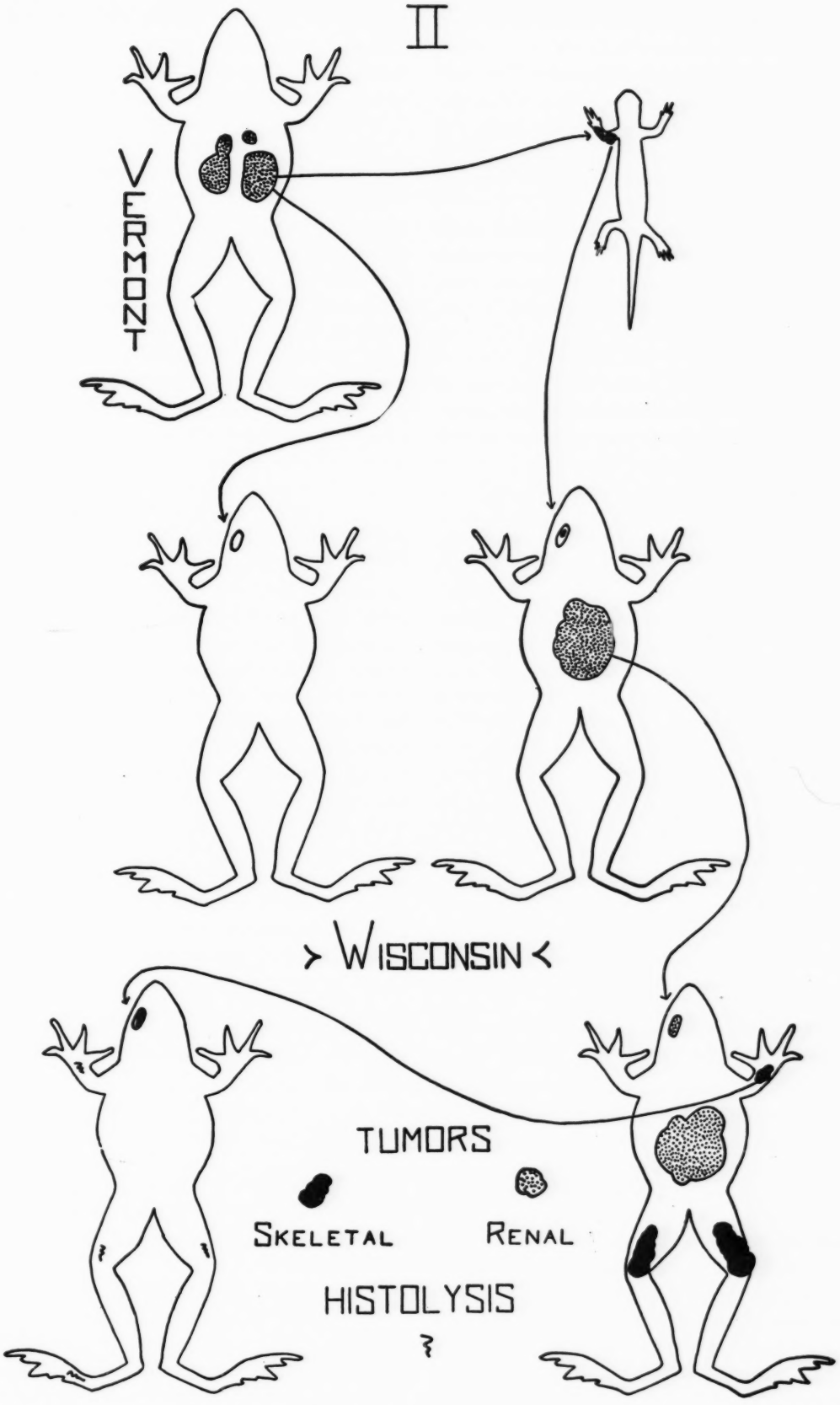


CHART 2.—Summary of tumors induced after transfer of a frog renal tumor agent to cartilage of a young salamander.

very cloudy, and iris tissue is very likely to invade the transplanted tumor within a few days when a Vermont to Wisconsin transfer is made. The intimate contact of host iris cells and implanted tumor is usually followed by destruction of the tumor cells, and no evidence of the release of an agent which will induce tumors in Wisconsin *R. pipiens* has been obtained.

A Vermont renal carcinoma (Fig. 14), the same one which was used in the transfer to efts, was also transplanted to the eye chambers of frogs from Wisconsin. Within 2 weeks iris tissue had completely encapsulated the tumor grafts. One was extracted 1 month after implantation in an eye from a Wisconsin frog and transferred to eyes of frogs from Vermont. In one case, after almost complete regression, rapid growth was resumed in one eye chamber of a Vermont frog (Fig. 21), but the tumor had changed. Instead of containing only the colorless elements of the original renal carcinoma, it now contained black cells as well, giving the growing tumor a gray appearance. A biopsy from the eye chamber is shown in Figure 24. The complex epithelium of the renal carcinoma now has, growing along with it, and as rapidly, pigmented cells of the iris. Small bits of this combination of renal carcinoma and rapidly growing iris elements were transplanted to some eye chambers of Wisconsin frogs. There was a strong reaction to the graft, and the implants in the left eyes had almost completely regressed within 2 weeks. In one to be considered further, a scar in the cornea through which the implant had been made was still visible, and a tiny piece of encapsulated tumor remained on the iris of the left eye at the end of 2 weeks; but a rapidly growing tumor was already apparent in the right eye. This eye had not been operated upon. The tumor arising either in the right iris or in an adjacent tissue was invasive and histolytic and reached the surface of the body between the eye and the ear (Fig. 22). This, like all the other tumors induced in tissues other than renal by a modified Lucké agent, grew very rapidly, was invasive, and finally became hemorrhagic and regressed. The cells of this particular tumor all died within a few days of one another. The tumor collapsed 14 days after it appeared, and the frog is still alive over a year later.

This was the sixth time that a rapidly growing tumor had arisen contralateral to a point where a tumor had been induced by a renal carcinoma agent. In all, including even doubtful cases, we have not seen more than 55 tumors induced in nonrenal tissues by the Lucké agent. The fact that 6 have been followed by secondary contralateral growth is significant. In all cases the nonrenal tumors have grown more rapidly than renal tumors, their morphological appearance has been more abnormal, they have been more invasive and hemorrhagic, and have regressed within 3 months unless they have killed the host in the meantime.

DISCUSSION AND CONCLUSIONS

Although the biochemical nature of the Lucké agent is not yet known, other tumor agents are or contain nucleoproteins (4, 7, 8, 22). Genetically marked, closely related nucleoprotein systems, wherever they have been grown together in the same cell, have been shown to possess the ability to interchange parts, and new combinations have been produced. These new combinations can reproduce true to type. This is true in chromosomes and in bacterial viruses (9, 14, 18, 19). It is also known that a viable myxoma agent can be recovered following the transfer of something between an untreated fibroma and a heat-inactivated myxoma agent or "virus," as it is usually called. This Berry-Dedrick (3) transformation involves the transfer of something between two immunologically similar tumor agents. The present work is interpreted as representing the transfer between tumor agents and particles of normal cells leading to the formation of new tumor agents with affinity for their tissues of origin. It is to be remembered that similarity in size, in chemical nature, and in antigenic nature has already been demonstrated between tumor microsomes and microsomes of normal tissue (1, 2, 4, 7, 8, 22).

Inferences drawn from this work are that there must be particles with which the Lucké agent will combine which are different in different tissues and also within rather small regions of those tissues. If there are not such particles, it is difficult to understand how the transformed renal agent, after it has grown in chondrocytes, can affect chondrocytes of the identical region on the other side of the body. The new agent must be composed of two parts, one with affinity for renal epithelium and one for cartilage. The acquired affinity for cartilage came while the renal agent was a part of regenerating chondroblasts in an adult salamander or in chondrocytes or chondroblasts of young salamanders. The acquired affinity for cartilage may be retained while the agent is subsequently growing in renal tissue.

Other cases of transfer of the activity of the tumor agent from one tissue to another, besides the Duran-Reynals cases, are the Oberling-Guerin case (21) of agent transfer from leukocytes to connective tissue and the Burmester-Prickett-Belding case (6) of transfer between lymphoidal and skeletal tissues.

Since transfer and formation of new viable combinations are only known to occur between closely related nucleoproteins, the suggestion is made that original tumor agents are modified tissue-differentiated nucleoproteins. An unmodified Lucké agent, according to this point of view, would be a modified kidney epithelium particle. This interpretation would include, then, both the somatic mutation hypothesis of tumor formation and the virus hypothesis. A tumor virus or agent would be a mutated normal tissue-specific particle. To be recognized as a tumor agent it would have to retain its activity when introduced into cells of another host. It would also have to direct differentiation toward cell types which are not so abnormal that they cannot continue to grow. Toward the end of both series of transfers reported above, the agents had been so transformed that cells harboring them were not viable for longer than a few days or at the most a few months. By that time they were more in the nature of histolytic agents than tumor agents.

The hypothesis presented here of the nature of the tumor agent is similar to that of Oberling (21). There is also similarity to the suggestion by R. G. Green (13) that, during tumor cell formation, virus and cell component may hybridize, giving a new type of self-reproducing agent. The hypothesis presented here differs in that, although it does not exclude the possibility that an extraneous nucleoprotein may sometimes be involved, such an addition is not considered necessary for neoplastic transformation. A more complete discussion of the relationship of the present findings to the modern hypotheses of cellular differentiation will be published elsewhere.

SUMMARY

1. The Lucké tumor agent, an agent with the ability to enter and grow in cells of only one differentiated tissue of only one race of frogs, has been changed by incorporation either in cells of young salamanders or in regenerating cells of adult salamanders. The new agents show affinity when returned to frogs for both the original host tissue, renal epithelium, and for their new host tissue, skeletal tissue.

2. Transfer of the renal agent, or a part of it, to the iris was demonstrated during tumor transplantation between different geographical varieties of the same species.

3. Evidence suggesting that a tumor agent may combine with particles which are different in different regions within a tissue is presented. The degree of regional differentiation is so great that secondary tumors frequently appeared in the region bilaterally identical to the site of the primary tumor formation. It is suggested that the data indicate the possibility of the existence of particles which are different in the various tissues and with which the tumor agents can combine in a process similar to crossing-over of genes to produce new types of tumor agents with affinities for their tissues of origin.

REFERENCES

1. AMIES, C. R. The Particulate Nature of Avian Sarcoma Agents. J. Path. & Bact., **44**:141–66, 1937.
2. AMIES, C. R.; CARR, J. G.; and LEDINGHAM, J. C. G. The Filterable Agents of Avian Sarcomata and Serological Experiments in Connection Therewith. Rep. 3d Intern. Cong. for Microbiology, p. 335, 1940.
3. BERRY, G. P., and DEDRICK, H. M. A Method for Changing the Virus of Rabbit Fibroma (Shope) into That of Infectious Myxomatosis (Sanarelli). J. Bact., **31**:50–51, 1936.
4. BITTNER, J. J. Some Enigmas Associated with the Genesis of Mammary Cancer in Mice. Cancer Research, **8**:625–39, 1948.
5. BRIGGS, R. Transplantation of Kidney Carcinoma from Adult Frogs to Tadpoles. Cancer Research, **2**:309–23, 1942.
6. BURMESTER, B. R.; PRICKETT, C. O.; and BELDING, T. C. A Filtrable Agent Producing Lymphoidal Tumors and Osteopetrosis in Chickens. Cancer Research, **6**:189–96, 1946.
7. CLAUDE, A. Particulate Components of Normal and Tumor Cells. Science, **91**:77–78, 1940.
8. CLAUDE, A., and ROTHEN, A. Properties of the Causative Agent of a Chicken Tumor. XIV. Relation between a Tumor Nucleoprotein and the Active Principle. J. Exper. Med., **71**:619–33, 1940.
9. DELBRUCK, M., and BAILEY, W. T., JR. Induced Mutations in Bacterial Viruses. Cold Spring Harbor Symp. Quant. Biol., **11**:33–37. 1946.
10. DURAN-REYNALS, F. The Reciprocal Infection of Ducks and Chickens with Tumor-inducing Viruses. Cancer Research, **2**:343–69, 1942.
11. ———. The Infection of Turkeys and Guinea Fowls by the Rous Sarcoma Virus and the Accompanying Variations of the Virus. *Ibid.*, **3**:569–77, 1943.
12. Foulds, L. The Growth and Spread of Six Filterable Tumours of the Fowl, Transmitted by Grafts. Eleventh Scientific Report, Imperial Cancer Research Fund, pp. 1–13, 1934.
13. GREEN, R. G. Parasitic Hybridization as a Cause of Cancer. Biodynamica, **6**:1–21. 1946.
14. HERSHEY, A. D., and ROTMAN, R. Genetic Recombination between Host-range and Plaque-type Mutants of Bacteriophage in Single Bacterial Cells. Genetics, **34**:44–71, 1949.
15. LUCKÉ, B. A Neoplastic Disease of the Kidney of the Frog, *Rana pipiens*. Am. J. Cancer, **20**:352–79, 1934.
16. ———. Carcinoma in the Leopard Frog: Its Probable Causation by a Virus. J. Exper. Med., **68**:457–68, 1938.
17. LUCKÉ, B., and SCHLUMBERGER, H. Neoplasia in Cold-blooded Vertebrates. Physiol. Rev., **29**:91–126, 1949.
18. LURIA, S. E. Reactivation of Irradiated Bacteriophage by Transfer of Self-reproducing Units. Proc. Nat. Acad. Sc., **33**:253–64, 1947.

19. Luria, S.E. Bacteriophage: An Essay on Virus Reproduction. Science, **111**:507–11, 1950.
20. Murphy, J. B. Experimental Approach to the Cancer Problem. Bull. Johns Hopkins Hosp., **56**:1–31, 1935.
21. Oberling, C. The Riddle of Cancer, pp. 140–41, 157. New Haven: Yale Univ. Press, 1944.
22. Pollard, A. The Chemical Composition of the Active Agent of the Rous Sarcoma No. 1 and Some Related Products. Brit. J. Exper. Path., **20**:429–38, 1939.
23. Rose, S. M.; Rose, F. C.; and Wallingford, H. M. Methods of Establishing the Lucké Carcinoma in *Triturus viridescens*. Anat. Rec., **108**:622, 1950.
24. Rous, P. A Sarcoma of the Fowl Transmissible by an Agent Separable from the Tumor Cells. J. Exper. Med., **13**:379–411, 1911.
25. Rous, P., and Lange, L. B. The Characters of a Third Transplantable Chicken Tumor Due to a Filterable Cause. A Sarcoma of Intracanicular Pattern. J. Exper. Med., **18**:651–64, 1913.
26. Rous, P., and Murphy, J. B. On the Causation by Filterable Agents of 3 Distinct Chicken Tumors. J. Exper. Med., **19**:52–69, 1914.
27. Rous, P.; Murphy, J. B.; and Tytler, W. H. A Filterable Agent the Cause of a Second Chicken-Tumor, an Osteochondrosarcoma. J.A.M.A., **59**:1793–94, 1912.
28. Schlumberger, H. G., and Lucké, B. Tumors of Fishes, Amphibians, and Reptiles. Cancer Research, **8**:657–753, 1949.

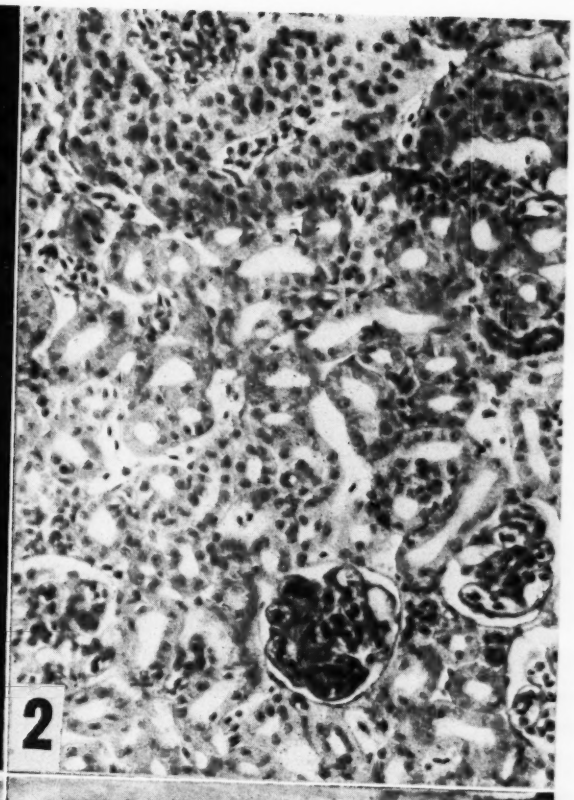
Fig. 1.—*Rana pipiens* with two large loci of carcinoma in right kidney and three smaller loci, surrounded by oviduct, in left kidney.

Fig. 2.—Junction of carcinoma and normal renal tissue. Renal capsules and normal tubules below, cords of carcinoma and stroma above. ×135.

Fig. 3.—21-Day graft of renal carcinoma of frog in salamander limb. The several larger nuclei are of the salamander host. ×550.

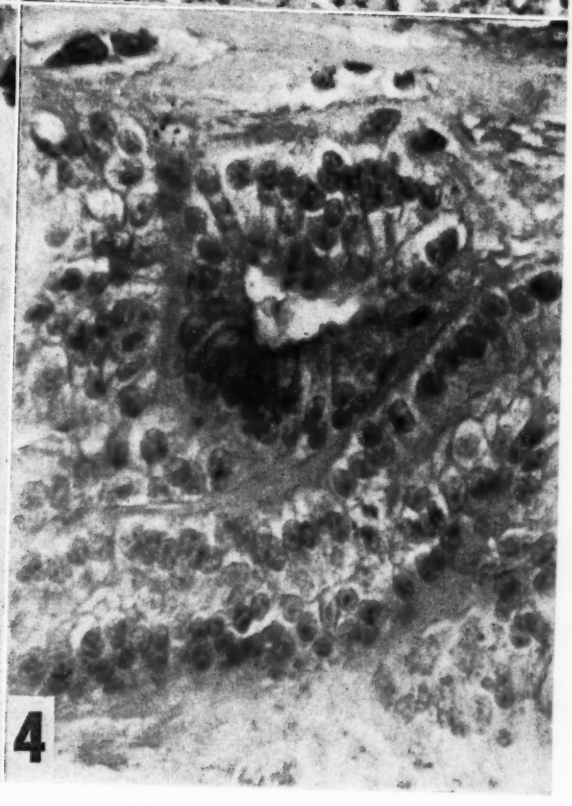
Fig. 4.—92-Day graft of renal carcinoma of frog in salamander limb. ×200.

1



2

3



4

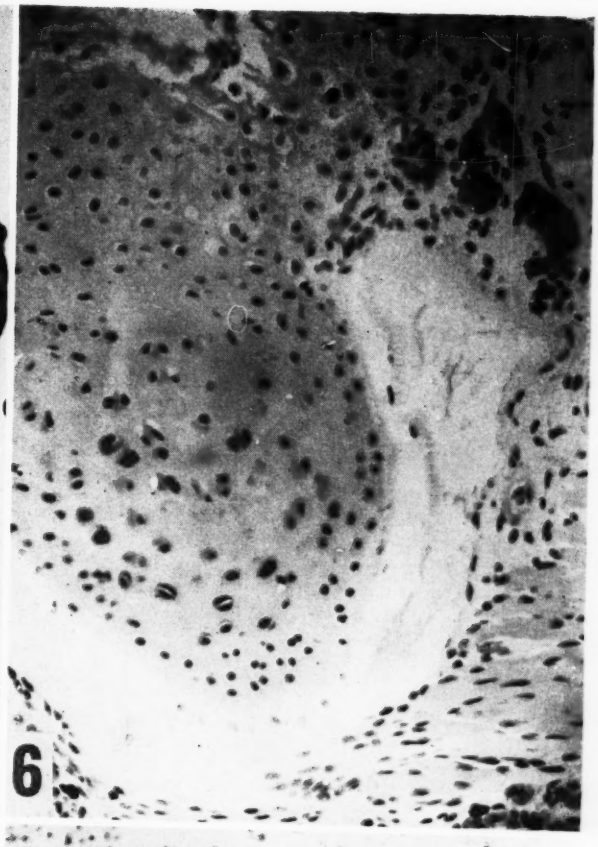
FIG. 5.—Renal carcinoma of frog established in subcutaneous area of limb of salamander. ×125.

FIG. 6.—Junction of chondrosarcoma and normal cartilage in limb of salamander. The more irregular chondrosarcoma above connects with distal head of humerus in center. ×100.

FIG. 7.—Same limb as in Figure 5, but after regeneration blastema had formed. All old tissues, both normal and neoplastic, replaced by regenerating cells; new cartilage forming at base of photograph. ×100.

FIG. 8.—Osteogenic region of tumor from femur of frog. ×200.

5



6

7

8

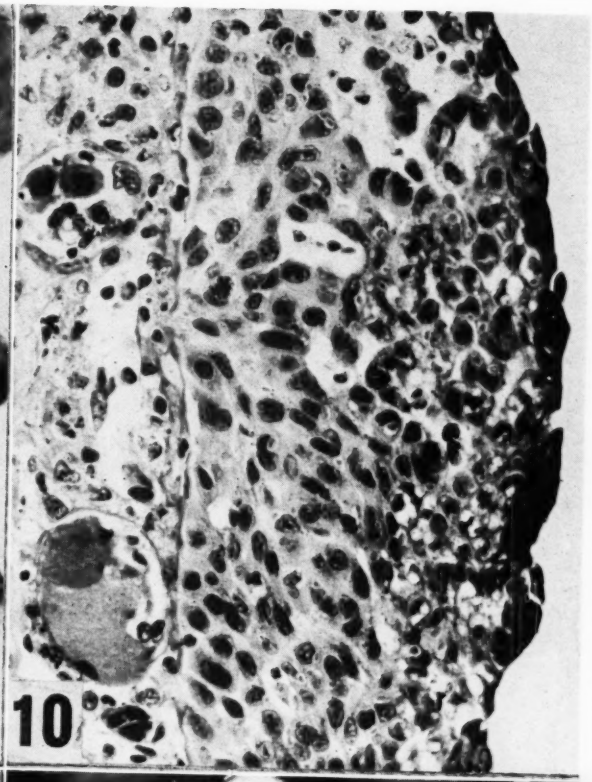
FIG. 9.—Anaplastic tumor which had spread subcutaneously in young salamander from forearm to cervical region after a Lucké renal carcinoma had been implanted in the forearm. ×150.

FIG. 10.—Anaplastic tumor in subcutaneous region of head of young salamander. ×150.

FIG. 11.—Histolytic region of limb regeneration of eft of Triturus. ×950.

FIG. 12.—Chondrosarcoma induced by renal carcinoma of frog in left forelimb of eft of Triturus. ×3½.

9


10

11

12

FIG. 13.—The source tumor used in the first series involving transfer of agent to regenerating salamander cells. ×135.

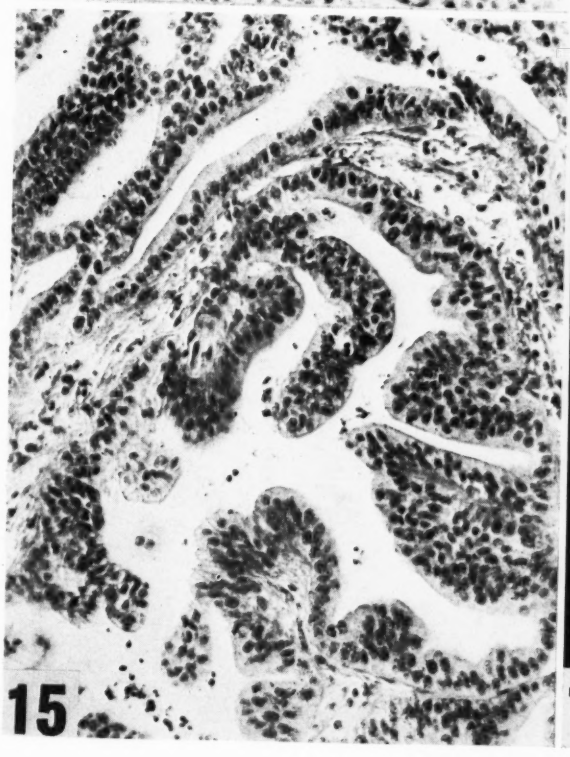
FIG. 14.—The source tumor used in the second series involving transfer of agent to young salamander cells. ×175.

FIG. 15.—Renal carcinoma which arose in kidney of frog following implantation of chondrosarcoma of salamander. ×135.

FIG. 16.—Osteogenic tumor growing in anterior chamber of the eye of a frog.

13

14



15

16

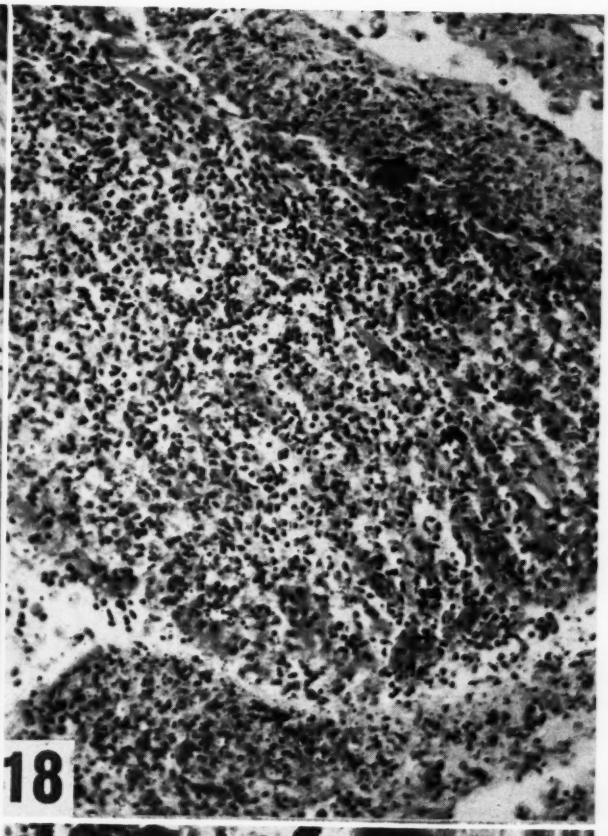
FIG. 17.—Regenerated elbow region of Triturus limb which had had a chondrosarcoma at amputation level at time of amputation. ×100.

FIG. 18.—Nodule in mesentery induced by modified agent from chondro-osteosarcoma. ×150.

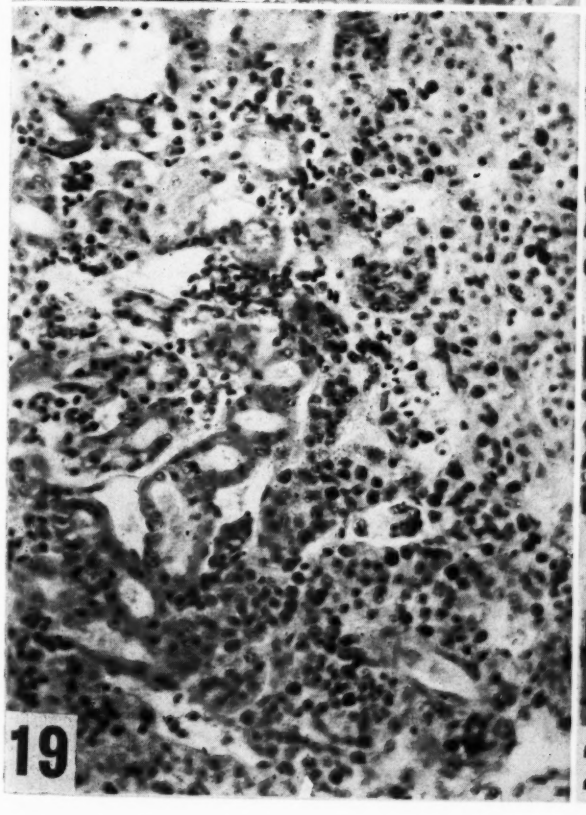
FIG. 19.—Tumor in kidney induced by modified agent from chondro-osteosarcoma. ×150.

FIG. 20.—Sarcolysis in muscle of leg following abdominal injection of modified agent from chondro-osteosarcoma. ×400.

17



18



19

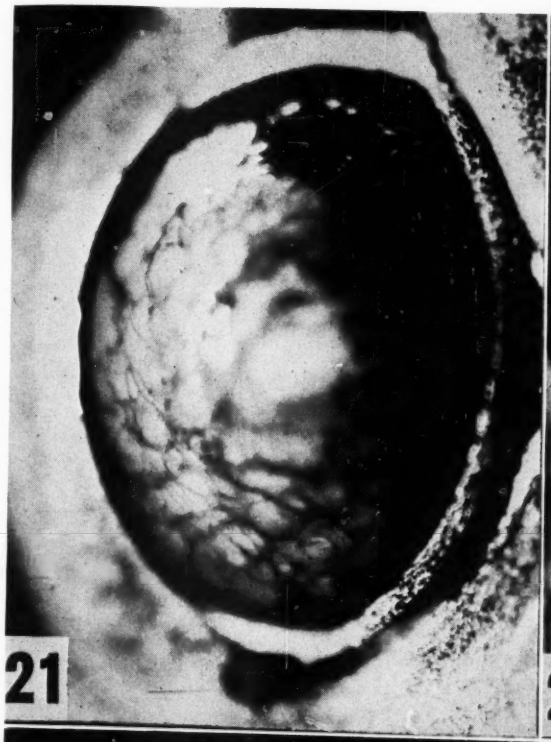
20

FIG. 21.—Combination renal and iris tumor in anterior eye chamber of frog. ×10.

FIG. 22.—Eye tumor reaching surface between right eye and ear. This tumor had arisen in right eye after an implant of a piece of the tumor of Figures 21 and 24 had regressed in left eye.

FIG. 23.—Hemorrhagic lesions in right and left sartorius muscles induced by implant to eye chamber of skeletal tumor of wrist.

FIG. 24.—Second growth of renal carcinoma plus pigmented iris elements in the anterior chamber of the eye. ×150.


21

22

23

24

The Action of Tumors on Normal Tissue Synchronously Transplanted into the Anterior Chamber of the Mouse Eye*

HENRY BROWNING

(Department of Anatomy and the Laboratory of Pathology, Yale University School of Medicine, New Haven, Conn.)

INTRODUCTION

Greene and co-workers showed that tumors of man and other mammals may be transplanted into the anterior chamber of the eyes of alien species (12–15). The fate of such heterologous transplants varied from early regression to progressive growth. When spontaneous mammary adenocarcinomas of mice were transferred to alien strains, the results varied with the stage of development (3). The ability to grow on transfer increased as the tumors progressed and was at a maximum in large solitary tumors or in metastases. In contrast to some of the results of Greene, no transplant survived indefinitely or grew progressively. Regression always occurred, usually shortly after the tenth day following transplantation.

In general, the tissues of newborn mice survived indefinitely without growth on homologous transfer (4). When such transplants were in contact with heterologous mammary gland tumor in the same eye, there was often very considerable growth of the complex. The grafts grossly resembled tumor rather than neonatal tissue and persisted indefinitely. The present report concerns this type of reaction between neoplastic and normal mouse tissues. As previously (3, 4), the term heterologous has been applied to transfers between different mouse strains and the term homologous to transfers within the same strain.

MATERIALS AND METHODS

Spontaneous mammary adenocarcinomas occurring in the breeding females of C3H, DBA, or first generation hybrids of these strains were used for transplantation. Unless otherwise stated, normal tissues were derived from newborn C57 brown mice.

* This investigation was supported by grants from the American Cancer Society; the National Cancer Institute, U.S. Public Health Service; the James Hudson Brown Memorial Fund; and the Jane Coffin Childs Fund for Medical Research.

Received for publication July 25, 1951.

Control transplants of normal tissue (homologous) and of tumor tissue (heterologous), and dual transplants of normal (homologous) and tumor (heterologous) tissues were made into C57 brown mice. Control homotransplants of tumor were made into mice of the strain of tumor origin. Host mice were 2–4 months old and of either sex. The progress of the transplants was determined by scale drawings under a dissecting microscope every 7 days during the first month and then at monthly intervals. All types of transplants, when removed for histological study or at termination of an experimental series, showed volumetric size in proportion to the surface area directly measurable.

The behavior of control homotransplants of normal tissues confirmed previous observations (4). Lung, thymus, and spleen survived indefinitely without change of size; kidney and cerebral cortex survived indefinitely after considerable absorption; and liver was completely absorbed.

The control heterotransplants of tumor gave an estimate of its autonomy—or ability to grow in an alien environment. As noted previously (3), tumor tissue that failed to grow was considered to be without autonomy; that which increased in surface area up to 3 times was called mildly autonomous; and that which similarly grew to 4–6 times, fully autonomous. The latter amount of growth was the maximum observed, and all heterotransplants regressed by the fourteenth day after transfer. Usually solitary tumors of less than 1 cm. in average diameter were nonautonomous; those between 1 and 2 cm., mildly autonomous; and those over 2 cm., fully autonomous. Control homotransplants established the viability of nonautonomous tumors.

For each spontaneous tumor a series of control and dual transplantations were made. Basically, in any one series each type of transfer was made to seven or eight hosts, both eyes being used. Thus, fourteen to sixteen transplants of each type were

observed throughout the experimental period. However, dual transplants were also made into sufficient additional hosts to allow the removal of two to four transplants for histological study at most observations.

The technic of intraocular transplantation (3) was modified by filling the trocars used with molten 2 per cent agar jelly. When cool, most of the column of solidified jelly in the lumen was expressed. The short segment left acted as an efficient plunger and prevented adhesion of tissue to the stylet. In most dual transplantations both tissues were taken into the same trocar and introduced into the eye in a single operation, thus avoiding a repetition of trocar introduction that tended to give infection. When the two tissue fragments were to be kept separate in the eye an intervening air bubble was included between them in the trocar.

TABLE 1

AUTONOMY OF TUMOR TRANSPLANTS AND GROWTH OF TUMOR-LUNG TRANSPLANTS AFTER 2 WEEKS AND AFTER 3 MONTHS

		Growth of tumor-lung transplant at		Tumor characteristics		
Series	Autonomy of tumor	14th day	90th day	Strain of origin	Solitary or multiple	Av. diameter (cm.)
1	none	x 1	x 1	C3H	solitary	0.5
2	none	x 1½	x 1	C3H	solitary	0.6
3	none	x 1½	x 1	C3H	multiple	0.4
4	none	x 2	x 1	C3H/DBA	solitary	1.0
5	mild	x 2	x 1	DBA	solitary	2.0
6	mild	x 2½	x 1	DBA	solitary	2.0
7	mild	x 3	x 1	C3H	solitary	2.0
8	mild	x 3	x 1	C3H	solitary	0.7
9	mild	x 3	x 1	C3H/DBA	solitary	1.0
10	mild	x 3½	x 1	C3H/DBA	solitary	1.0
11	full	x 4	x 2	C3H	multiple	1.7
12	full	x 6	x 3	C3H	solitary	2.0
13	full	x 6	x 4	C3H	solitary	2.5
14	full	x 6	x 4	C3H	solitary	2.7

RESULTS

Tumor and Newborn Lung

Fourteen tumors were used for the control and dual transplantations with newborn lung. In Table 1 the results are expressed as modal averages of growth of dual transplants and are grouped into three categories, corresponding to the autonomy of the tumor. In contrast to solitary heterotransplants of tumor, there was little variation from the mode.

In the first category (series 1–4), the tumor and lung tissue in dual transplants were partially merged by the seventh day after transfer. By the fourteenth day, both grossly and microscopically, only lung remained. Its fragments were often slightly enlarged but reverted to their original dimensions by the 21st day, after which they persisted indefinitely without change.

In the second group (series 5–10), tumor and lung in dual transplants merged completely by the seventh day, and the complexes had doubled in size. By the fourteenth day there was usually further growth to 3 times the original size. On the 21st day there was no further change, but by the end of the first month the complexes had decreased to 1½–2 times the original size. By the end of the third month the complexes had diminished to the size of the control lung transplants.

The progress of the dual transplants in the third group (series 11, 12, 13, 14) was similar. However, the complexes had increased in size by 3–4 times on the seventh day after transfer and by 4–6 times on the fourteenth day. They showed slight reduction in size by the end of the first month and but little thereafter. By the end of the third month they had an average size of 3 times the original transplant and then persisted indefinitely without change.

In some cases (series 7, 9, 10, 12, and 15), tumor-lung transplants showing conspicuous growth after the fourteenth day were removed from half of the animals in the respective series and introduced into the eyes of mice strains homologous for the tumor component. No growth occurred. Evidently no living tumor was present in the complexes after the fourteenth day in the original hosts.

Histologically, the tumor-lung transplants showed tumor cells actively invading the lung tissue on the seventh day after transfer, with a lymphocytic infiltration at the interface of the two tissues (Fig. 1). By the tenth day the lymphocytic infiltration had increased, and only a few tumor cells were visible. No tumor cells were seen on or after the fourteenth day (Fig. 2). The increase in

size of the transplants at this time was partly due to the presence of the lymphocytes and partly to the growth of the lung tissue. The lymphocytic reaction diminished with the size of the transplants, but traces of it remained in the surviving lung tissues even after 3 months. Otherwise, no transplant differed histologically from control lung transplants, whether with or without permanent growth at this time (Fig. 3).

Tumor and Tissues Other Than Lung

Seven tumors were used for control and dual transplantations where the newborn tissue was thymus, spleen, kidney, cerebral cortex, or liver (Table 2). All tumors were mildly autonomous. Dual transplantations were also made of each tumor and newborn lung.

The growth of thymus and spleen tissues in dual transplants was identical with that of lung.

The reaction of kidney tissue with tumor was similar to that of lung. Control homotransplants showed considerable absorption, resulting in a thin plate of persistent kidney usually less than a quarter the size of the original fragment. With autonomous tumors the fragments not only did not diminish in size but grew to twice the original dimensions.

The reaction of cerebral cortex with tumor was less than that of any other tissue studied except liver. In control homotransplants the fragments persisted after partial absorption at approximately half the original size. With autonomous tumor little growth and no absorption occurred. The fragments reached a size of $1\frac{1}{2}$ times the original at the most.

Histologically, the persistent kidney fragments showed small areas of lymphocytic infiltration, but this never occurred to the extent seen in the lung complexes. No such infiltration was found in cerebral cortex complexes, and no such infiltration could be detected in spleen or thymus complexes.

No growth occurred in tumor-liver transplants. After the fourteenth day the complexes persisted indefinitely at the original size. Histologically, all other complexes showed the newborn tissues to have recognizable development and differentiation along adult lines; in the case of liver, the persistent tissue was fibroblastic and contained no liver cells (Fig. 4). No lymphocytic reaction was found. Control homotransplants of liver were completely absorbed by the fourteenth day after transfer.

Tumor and Embryonic, Newborn or Adult Lung

Seven tumors were used for control and dual transplantations with mouse lung at various stages of development (Table 3).

In series 22 and 23 tumor was transplanted with lung from 16-day embryonic, newborn, or adult C57 brown mice. The growth of the complexes was from 4 to 6 times, 2 to 3 times, and 2 times, respectively. Control homotransplants of 16-day embryonic lung grew to twice their original size; those of newborn and adult lung persisted without growth. Allowing for this growth of embryonic lung itself, it appeared that the reaction between lung and tumor was not related to the developmental stage of the former. In series 24, newborn and adult lung gave parallel results with a fully autonomous tumor.

In series 25 and 26, fragments of newborn or adult lung were introduced into the host eyes 1 week before addition of the tumor; newborn lung alone gave the usual reaction. In series 27 and 28, fragments of newborn or adult lung were transplanted 1 month before the tumor; neither lung tissue reacted.

Tumor and Nonadjacent Newborn Lung Tissue

Two mildly autonomous tumors were used for control and dual transplantations where the lung and tumor fragments were introduced into the same eye but placed at opposite poles of the anterior chamber (series 29 and 30, Table 4). Dual transplants were also made of the same tissues in the usual adjacent positions.

TABLE 2

AMOUNT OF GROWTH OF DUAL TRANSPLANTS OF MILDLY AUTONOMOUS TUMOR WITH VARIOUS NEWBORN TISSUES BETWEEN 2 AND 3 WEEKS AFTER TRANSPLANTATION

	Growth of tumor-tissue complex between 14th and 21st days						Tumor characteristics		
Series	Lung	Thymus	Spleen	Kidney	Cerebral cortex	Liver	Strain of origin	Solitary or multiple	Av. diameter (cm.)
15	x 3	x 3			x $1\frac{1}{2}$		C3H/DBA	solitary	1.0
16	x 4		x 4		x $1\frac{1}{2}$		C3H	solitary	1.7
17	x 3			x 2		x 1	C3H/DBA	solitary	1.0
18	x 3			x 2		x 1	DBA	solitary	2.0
19	x 3			x 2		x 1	DBA	solitary	2.0
20	x 4	x 4	x 4		x $1\frac{1}{2}$		C3H	multiple	1.7
21	x 3	x 3	x 3				DBA	solitary	1.8

Nearly all the lung fragments in the eyes containing a tumor at the opposite poles showed an equal or even greater reaction than those in adjacent transplantation. They grew to 3 or even 4 times the size of the original transplant, while the adjacent tissues increased but 2–3 times. Histologically, such fragments showed early invasion by tumor cells and a course identical with adjacent tumor-lung transplants. Occasionally, tissue strands crossing the iris from tumor to lung could through wire gauze. Equal parts of the resultant mash and 2 per cent agar jelly were mixed at 40° C. and introduced into trocars. Fragments of the solidified jelly were transplanted with adjacent lung fragments. The size of the fragments of jelly was equal to or larger than the tumor fragments usually employed. No reaction occurred in the lung tissue. No growth occurred in control homotransplants of jelly fragments. Histologically, these fragments contained viable cells and cell

TABLE 3

AMOUNT OF GROWTH OF TUMOR-LUNG COMPLEXES WHEN THE LUNG COMPONENT VARIED IN MATURITY OR WHEN THE TWO COMPONENTS WERE NOT TRANSPLANTED SIMULTANEOUSLY

Series	Autonomy of tumor	Growth of tumor-lung complex between 14th and 21st days when lung derived from: Embryo	Newborn	Adult	Interval between transplantation of lung and tumor	Tumor characteristics: Strain of origin	Solitary or multiple	Av. diameter (cm.)
22	mild	x 4–6	x 2–3	x 2	nil	C3H	solitary	1.3
23	mild	x 4	x 2	x 2	nil	DBA	solitary	1.8
24	full		x 4–6	x 4–6	nil	C3H	solitary	2.6
25	full		x 4	x 1	1 week	DBA	solitary	3.0
26	mild		x 2–3	x 1	1 week	C3H/DBA	solitary	1.4
27	full		x 1	x 1	1 month	C3H	multiple	2.2
28	mild		x 1	x 1	1 month	C3H	solitary	1.6

TABLE 4

AMOUNT OF GROWTH OF COMPLEXES OF TUMOR WITH ADJACENT AND NONADJACENT LUNG AND OF INTACT AND NONINTACT TUMOR WITH ADJACENT LUNG

Series	Autonomy of tumor	Growth of tumor-lung complex between 14th and 21st days: Lung adjacent	Lung non-adjacent	Tumor intact	Tumor non-intact	Tumor characteristics: Strain of origin	Solitary or multiple	Av. diameter (cm.)
29	mild	x 2	x 2–4			C3H	solitary	1.0
30	mild	x 2	x 2–3			C3H	solitary	2.0
31	full			x 4	x 1	DBA	solitary	3.0
32	full			x 6	x 1	C3H	multiple	1.7
33	full			x 6	x 1	C3H	solitary	2.0
34	full			x 4–6	x 1	C3H	solitary	2.6

be seen grossly; no other contact was visible. In a few cases in each series (6 out of 34 transfers), there was no lung reaction.

Nonintact Tumor and Adjacent Lung

Four fully autonomous tumors were used for control and dual transplantations with newborn lung after interference with the integrity of the tumor (series 31–34, Table 4). The behavior of the intact tumor in dual transplantations had been determined in earlier series.

In series 31 and 32, the tumor tissue was stored in a deep freeze for 1 and 3 months, respectively. No reaction occurred between lung and fragments of these tumors, nor did control homotransplants of the tumor survive and grow. Before freezing, both tumors had given a marked reaction with lung (series 12 and 13, Table 1).

In series 33 and 34, the tumor tissue was forced clumps. The intact tumors had given a marked reaction with lung in series 11 and 14 (Table 1).

DISCUSSION

Heterologous mammary gland tumors evoked a growth reaction in normal tissues when both were transplanted to the anterior chamber of the eye. The occurrence, extent, and permanence of this growth were directly related to the autonomy of the tumor, autonomy being here defined as the ability to grow in an alien strain of host. The extent of the induced growth also varied with the normal tissue concerned, but the specificity of reaction was not of the order found by Levi-Montalcini and Hamburger (17) in the chick embryo, where certain sarcomas stimulated sympathetic and sensory cells and fibers alone. The capability for a growth reaction also varied indirectly with the maturity of the normal tissue, newborn lung

losing it relatively slowly after transplantation and adult lung relatively rapidly. The reaction required contiguity of the tumor and normal cells. The stimulating substances of the former appeared to be very labile or to have a short range of action, a suggestion emphasized by the lack of effect of dead or fragmented tumor.

The hypothesis is advanced that tumor tissues, once autonomy is attained, can stimulate connective tissue or mesenchymal cells with which they are in contact. Normally, this stimulation produces vascular and stromal response in a heterologous or homologous host. That such a response, at least of capillary endothelium, is evoked by tumor cells has been shown by Algire and Chalkley (1), although there are exceptions, as Williams (22) found for the V_2 carcinoma of Kidd and Rous. Furthermore, the vascular response to transplants from tissue culture parallels the capacity of the culture to give rise to sarcomas (Algire, Chalkley, and Earle [2]). Coman and Sheldon (7) also found that local hyperemia is induced by subcutaneous transplants of tumor or embryonic tissue but not by normal tissue.

In immature tissues this stimulation produces proliferation of the "stroma" or undifferentiated mesenchymal elements. Growth of the tissue follows in response to these augmented supporting cells. Fragments of adult tissues, displaced from their normal environment in the donor, induce the proliferation of mesenchymal cells in preparation for re-establishment. These cells, themselves virtually embryonic, respond to the stimulation of the tumor. In either case, the response is much more readily evoked from transplants than from the iris substrate that is not immediately endowed with large numbers of responsive cells. As the adult transplants become established, their recently proliferated cells are utilized, and responsiveness to the adjacent tumor disappears soon after transfer. Neonatal tissue is rich in such cells and reacts vigorously over a relatively long period until the tissue has become differentiated in its adult environment.

Such a hypothesis would account for the marked response of such tissues as lung, thymus, and spleen and for the moderate response of kidney. The poor response of the neuronal tissues might be expected, as they contain few mesenchymal cells capable of reacting. The "stromal" cells of liver also would be expected to respond, but the specific elements, requiring unknown conditions for proliferation (4), would be incapable of taking advantage of this elaboration of supporting elements.

Stimulating substances elaborated by tumor cells have been reported by numerous authors (Flexner and Jobling [11], Chambers and Scott [6], Martin and Barral [20], Casey [5], Snell, Cloudman, Failor, and Douglas [21], Kaliss, Jonas, and Avnet [16]). In general, these substances were present in dead and fractionated material and acted on the cells of the tumor of origin only. In this latter respect they differ from the hypothetical stimulating substance of the present work. Substances promoting fibroblastic proliferation in tissue culture have been found in extracts of embryonic and adult chick heart (Margoliash and Doljanski [19]) and in tumor tissue (Doljanski, Hoffman, and Tenenbaum [10], Cook and Walsh [9]), but in no case was there any excessive stimulation by tumor components as compared to those of normal tissues. On the other hand, Ludford and Barlow (18) found that fibroblasts in tissue culture showed 8–10 times their normal growth when placed between two explants of mammary carcinomas of various mouse strains. The fibroblasts were stimulated regardless of the normal tissue of origin, and this action of living tumor resembles that being reported here. The stimulating substances may be allied to the postulated wound hormone released by injured cells to promote local proliferation of mesenchymal cells (8).

At present, the nature of the stimulation of normal by neoplastic tissue remains problematical. Of more immediate interest is the association of the reaction with the progress of the spontaneous tumor and the appearance of autonomy in it. This development of a stimulating capacity offers further evidence that a neoplasm, although histologically a cancer, is biologically not static; and that the initiation of a spontaneous neoplasm is not the end-point of the malignant process, but the beginning.

SUMMARY

1. Spontaneous mammary gland tumors of inbred mice transplanted to the anterior chamber of eyes of alien strains stimulated growth of adjacent transplants of newborn lung if the tumors were autonomous.

2. Thymus and spleen from newborn mice were similarly stimulated, newborn kidney moderately, and newborn cerebral cortex only slightly. Newborn liver gave no growth reaction of its specific cells.

3. Embryonic, newborn, and adult lung were similarly stimulated if transplanted simultaneously with tumor. If transplanted 1 week before the tumor, only immature tissues reacted; if transplanted 1 month before, no reaction occurred in immature or mature tissue.

4. Stimulation of growth of newborn lung occurred when the tumor and normal tissue were not

in direct contact in the same anterior chamber.

5. Freezing or separation into cells and cell clumps destroyed the stimulatory capacity of the tumor.

6. It is suggested that the growth of normal tissues is due to the stimulation of their supporting elements, this reaction being of the same nature as that by which the tumor evokes a stromal and vascular response from host tissues.

REFERENCES

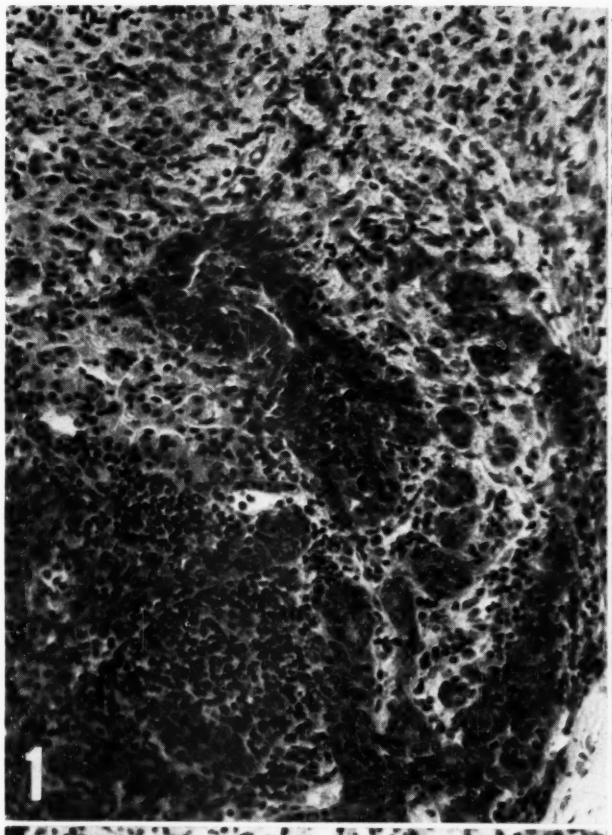
1. Algire, G. H., and Chalkley, H. W. Vascular Reactions of Normal and Malignant Tissues *in Vivo*. I. Vascular Reactions of Mice to Wounds and to Normal and Neoplastic Transplants. J. Nat. Cancer Inst., **6**:73–85, 1945–46.
2. Algire, G. H.; Chalkley, H. W.; and Earle, W. R. Vascular Reactions of Normal and Malignant Tissues *in Vivo*. III. Vascular Reactions of Mice to Fibroblasts Treated *in Vitro* with Methylcholanthrene. J. Nat. Cancer Inst., **11**:555–80, 1950–51.
3. Browning, H. C. Heterologous and Homologous Growth of Transplants during the Course of Development of Spontaneous Mammary Tumors in C3H Mice. J. Nat. Cancer Inst., **8**:173–89, 1947–48.
4. ———. Homologous and Heterologous Growth of Transplants of Various Tissues during the Course of development in the Mouse. Cancer, **2**:646–72;, 1949.
5. Casey, A. E. The Experimental Alteration of Malignancy with a Homologous Mammalian Tumor Material. II. Intracutaneous Inoculation of Preserved Material. Am. J. Cancer, **21**:776–80, 1934.
6. Chambers, H., and Scott, G. M. A Growth-promoting Factor in Tumor Tissue. Brit. J. Exper. Path., **7**:33–40, 1926.
7. Coman, D. R., and Sheldon, W. F. The Significance of Hyperemia around Tumor Implants. Am. J. Path., **22**: 821–25, 1946.
8. Cook, E. S., and Fardon, J. C. The Wound Hormone Concept in Wound Healing. Surg., Gynec. & Obst., **75**: 220–24, 1942.
9. Cook, E. S., and Walsh, T. M. The Effects of Extracts of Adult, Embryo and Tumor Tissues on the Growth of Yeast. Growth, **8**:251–58, 1944.
10. Doljanski, L.; Hoffman, R. S.; and Tenenbaum, E. The Effect of Tumor Extracts on the Growth of Cells *in Vitro*. Growth, **8**:13–31, 1944.
11. Flexner, S., and Jobling, J. W. On the Promoting Influence of Heated Tumor Emulsions on Tumor Growth. Proc. Soc. Exper. Biol. & Med., **4**:156–57, 1907.
12. Greene, H. S. N. Heterologous Transplantation of Mammalian Tumors. I. The Transfer of Rabbit Tumors to Alien Species. J. Exper. Med., **73**:461–74, 1941.
13. ———. Biological Differentiation of Benign and Malignant Growths. Bull. N.Y. Acad. Med., **20**:595–98, 1944.
14. Greene, H. S. N., and Lund, P. K. The Heterologous Transplantation of Human Cancers. Cancer Research, **4**: 352–63, 1944.
15. Greene, H. S. N., and Murphy, E. D. The Heterologous Transplantation of Mouse and Rat Tumors. Cancer Research, **5**:269–82, 1945.
16. Kaliss, N.; Jonas, G.; and Avnet, N. L. Growth Enhancement of Tumor Homoiotransplants in Mice Following Injections of Homogenates and Ultrafiltration Sediments of Mouse Tumors. Cancer Research, **10**:228, 1950.
17. Levi-Montalcini, R., and Hamburger, V. Selective Growth Stimulating Effects of Mouse Sarcoma on the Sensory and Sympathetic Nervous System of the Chick Embryo. J. Exp. Zool., **116**:321–62, 1951.
18. Ludford, R. J., and Barlow, H. The Influence of Malignant Cells upon the Growth of Fibroblasts *in Vitro*. Cancer Research, **4**:694–703, 1944.
19. Margoliash, E., and Doljanski, L. The Mitosis Stimulating Effects of Fresh Adult Chicken Heart Extract and Chick Embryonic Extracts. Growth, **14**:7–17, 1950.
20. Martin, J. F., and Barral, P. Reprise de la croissance du sarcome du rat à la suite d'injections d'extraits de ce même sarcome. Bull. Assoc. franç., l'étude cancer, **20**:370, 1931.
21. Snell, G. D.; Cloudman, A. M.; Failor, E.; and Douglas, P. Inhibition and Stimulation of Tumor Homoiotransplants by Prior Injections of Lyophilized Tumor Tissue. J. Nat. Cancer Inst., **6**:303–16, 1945–46.
22. Williams, R. G. The Vascularity of Normal and Neoplastic Grafts *in Vivo*. Cancer Research, **11**:139–44, 1951.

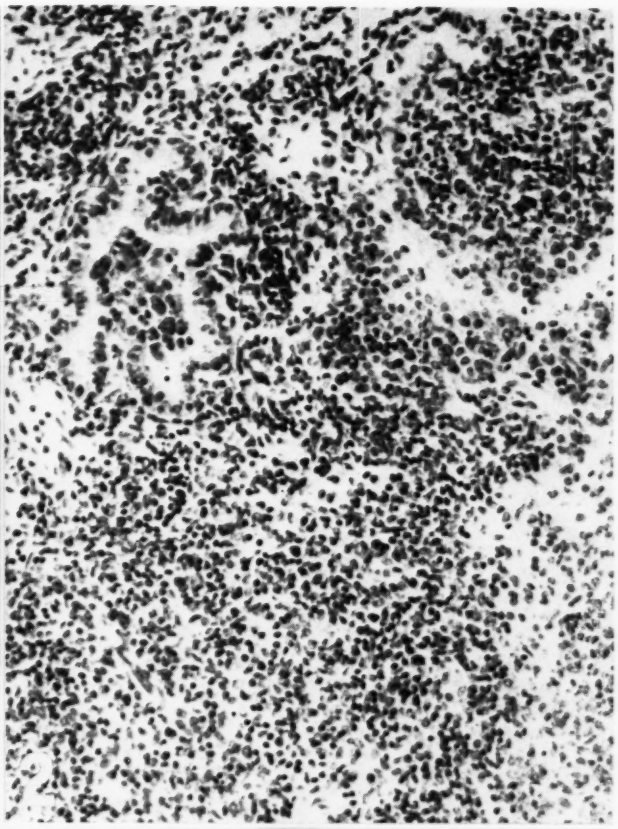
Fig. 1.—Tumor-lung transplant on 7th day; series no. 13; ×250. Invasion of lung by tumor with beginning lymphocytic proliferation at the interface of the two tissues.

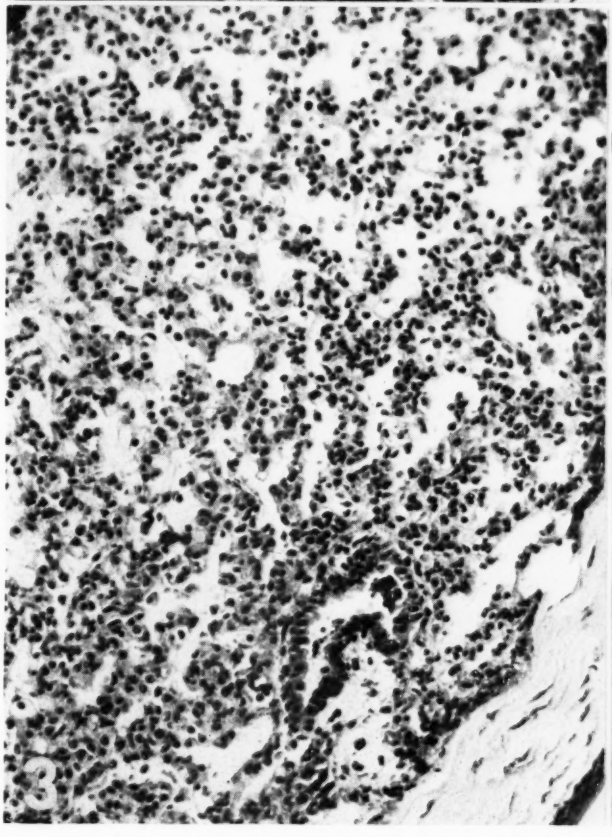
Fig. 2.—Tumor-lung transplant on 14th day; series no. 13; ×250. Lung tissue at peak of its growth with lymphocytic proliferation; tumor cells absent.

Fig. 3.—Tumor-lung transplant on 90th day; series no. 13; ×250. Persistent lung tissue with permanent growth.

Fig. 4.—Tumor-liver transplant on 21st day; series no. 19; ×200. Persistent fibroblastic tissue; tumor and liver cells absent.







A Mutation Producing Resistance to Several Transplantable Neoplasms in the C57 Black Strain of Mice*

PAULO R. F. BORGES AND BARBARA J. KVEDAR

(Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine)

Occasionally in the course of serial transplantations a tumor fails to grow progressively in animals of the inbred strain in which it originated, and this occurrence is generally interpreted as due to nongenetic factors. However, changes in the genetic constitution of the recipient hosts could be responsible for the resistance among certain animals of a susceptible strain. The purpose of this paper is to demonstrate such a possibility by showing that, by selective breeding, an isogenic resistant subline (26) was developed from the inbred C57 black strain of mice.

The C57 black strain (C57BL).—This strain was started by Dr. C. C. Little in 1921 from a heterogeneous stock of mice maintained by Miss A. E. C. Lathrop. A black male (No. 52) was mated to females No. 57 and 58 (his litter-mates) which were also black (14, 20). The descendants of female No. 57 segregated into blacks and brown, and from the black-colored mice the C57BL strain was developed (14). This strain has been inbred by brother-sister matings for more than 35 generations (15); therefore, it should be considered as having attained as high a degree of homozygosity as it is possible to obtain.

From the original C57BL strain a number of sublines have been separated since 1929. These sublines came from single pairs of mice brought from Ann Arbor, Michigan, to the R. B. Jackson Memorial Laboratory at that time (21). We have been unable to find out the exact number of sublines which have been developed or their characteristics. Little, Murray, and Cloudman (21) refer to sublines BIV, BV, BVI, BIX, X, CI, and CIII. At the present time only sublines 6 and 10 are maintained at this laboratory. Their responses toward transplanted tumors seem to be identical. The animals in both sublines are solid black (nonagouti), some yellowish hairs being present behind the ears and on the ventral aspect of the body, especially in the genital region. Occasionally, a few white hairs, or a definite white spot, are observed on the mid-line of the abdomen. Ventral spotting in mice apparently has a hereditary basis and probably is not determined by a single gene (25). The majority of the animals show white spots on the tail, such markings being due to a single recessive gene (11, 18). Additional information concerning the C57BL strain can be found in publications by Little, Murray, and Cloudman (21), Little (17), and Law (15).

The subline 10 of the C57 black strain (C57BL/10Sc or B10).—In 1940 Dr. J. P. Scott of the Hamilton Station of the Jackson Laboratory obtained pedigreed B10 mice from Dr. W. L. Russell, who at that time worked at the main laboratory building. Dr. Russell secured his animals from Dr. C. C. Little in 1937. The mice that Dr. Scott received from Dr. Russell had been inbred for at least twenty generations. Approximately eighteen more generations were obtained by Dr. Scott until 1948, when pedigreed pairs were supplied to Miss Judith H. Fielder of the Jackson Laboratory. Therefore, by 1948 the B10 subline had been inbred for at least 38 generations. At that time the colony maintained by Miss Fielder was turned over to one of us (P.R.F.B.). From this colony the isogenic resistant subline, temporarily called B10-*x*, was developed.

The development of the B10-x subline.—On November 2, 1948, experiments were started to test B10 mice for susceptibility to different mouse neoplasms, one of these being the C1498 myeloid leukemia. This leukemia originated in the subline 6 of the C57BL strain (B6) and had been carried by subcutaneous grafts for 224 transplant generations. It grew progressively in 100 per cent of both B6 and B10 mice, killing the hosts in 9–14 days, with a few animals dying during the third week after inoculation.

Leukemic tissue secured from the subcutaneous implants in B6 animals was grafted subcutaneous-

* This investigation was supported by a grant from the Charles R. Blakely Fund, administered by the National Research Council.

Received for publication September 4, 1951.

ly into B10 mice, a trocar being used for the inoculation. Whenever possible, five females and five males were employed in each experimental group, the age of the animals ranging from 4 to 6 weeks. Most of the inoculated mice came from our own colony of B10's (C57BL/10ScBs), but in two instances animals from other colonies were employed. After a number of trials in which the C1498 leukemia had been supplied by B6 mice, was the first time that B10 mice survived the inoculation of the C1498 leukemia. Of the seven resistant animals, three females (Nos. 171, 172, and 173) were mated to one male (No.180), and from their offspring the B10-*x* subline was developed. Females No. 172 and No. 173 died of nonleukemic causes at the age of 168 days (132 days after inoculation). Female 171 and male 180 were killed at the age of 449 days, after having produced most of

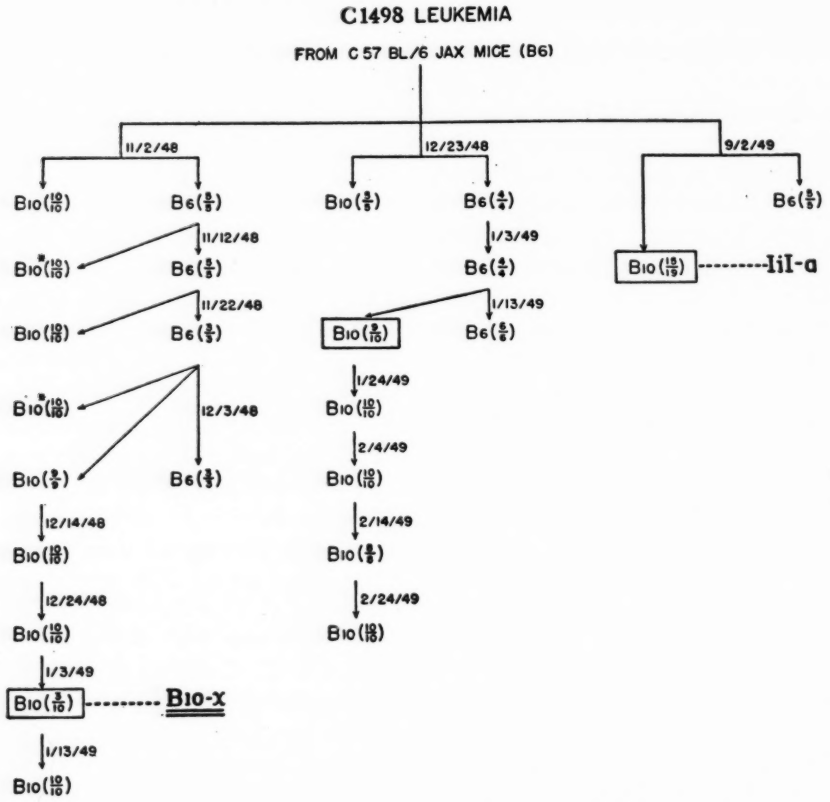


CHART 1.—Serial transplantations of the C1498 leukemia, showing the origin of sublines B10-*x* and 1i1-a developed by breeding resistant C57BL/10ScBs mice (B10).

Figures in parentheses: $\frac{\text{No. of susceptible mice}}{\text{No. of grafted mice}}$.

Asterisks indicate other colonies of C57BL/10Sc mice.

this neoplasm was transferred from B10 into B10 for two more transplant generations, all the twenty grafted animals proving to be susceptible (Chart 1).

On January 3, 1949, ten B10 animals (five females and five males), born around November 28, 1948, were grafted with the C1498 leukemia secured from a B10 mouse. The neoplasm grew progressively in three of the animals; one of them was killed after 10 days for a further transplantation into ten B10 mice, which proved susceptible. The other two animals died on the fourteenth day after inoculation. In the remaining seven mice (three females and four males) the subcutaneous tumor mass grew during the first week, then began to regress, and disappeared completely in 2–5 weeks. It

the animals in the B10-*x* colony. At the present time, the whole colony traces back to female 171 and male 180. Seven generations, comprising over 1,300 animals have been obtained by brother-sister matings.

Concerning coat color and other visible characters, the B10-*x* mice are identical with the B10 animals from which they were derived. So far, the only difference noticed lies in the manner in which they respond to the grafting of the C1498 leukemia and other neoplasms indigenous to the C57 strains (black and leaden) (Table 1). Every breeder in the B10-*x* colony has been inoculated with the C1498 leukemia and has proved to be resistant, a preliminary growth of the inoculum

being followed by complete regression in 3–4 weeks. A total of 612 mice has been tested; they were weaned at 4 weeks of age, and when 5–6 weeks old they were inoculated. On the other hand, the C1498 leukemia has been fatal to every mouse from the original B10 colony.

Other mice, resistant to the C1498 leukemia, found in the B10 colony.—On January 13, 1949, one out of ten grafted animals survived and was killed 77 days after inoculation. This mouse had been born around December 7, 1948. One of the susceptible animals was killed, and leukemic material secured from him was carried for four more transplant generations in B10 mice, all the 38 inoculated animals dying of leukemia (Chart 1).

On September 2, 1949, nineteen mice, born around June 6, 1949, were grafted with the C1498 leukemia. Of these, four (two females and two males) survived the experiment and were bred as a separate colony (1i1-a). Starting with the first filial generation, mice which had been previously grafted were not kept as breeders. This was done to compare this colony with the B10-*x* subline in which every mouse is born from previously grafted mothers. At the present time, seven generations of brother-sister matings have been obtained, and 144 mice, covering six generations, have been inoculated with the C1498 leukemia; all of them have survived.

Before the B10-*x* subline was started, groups of B10 mice had been saved to be used in an experiment concerning the age factor in relation to susceptibility to transplanted tumors. By October, 1949, ten groups of mice, born from September 16, 1948, to May 23, 1949, were grafted with the C1498 leukemia. Out of 116 animals, ten survived the inoculation. The results showed that as far back as September 16, 1948, before the B10-*x* subline was isolated (January 3, 1949), resistant mice existed in the B10 colony.

From November, 1949, to June, 1950, every breeding pair in the B10 colony was tested by inoculating their offspring with the C1498 leukemia; they were all susceptible, and no resistant animal has been found since.

MATERIALS AND METHODS

Most of the data presented in this paper were secured by inoculating different mouse tumors into sublines of the C57BL strain of mice. Mice of the dilute brown strain (DBA) were crossed with C57 blacks, and their F_1 hybrids were used in one of the experiments. All the animals were born and raised at the R. B. Jackson Memorial Laboratory, maintained on a diet of Purina Fox Chow pellets, supplemented with rolled oats once a week, and had an unlimited supply of water. Both the experimental and the control mice were kept under the same environmental conditions.

TABLE 1

COMPARATIVE STUDY OF THE SUSCEPTIBILITY AND RESISTANCE TO TRANSPLANTED MOUSE NEOPLASMS BETWEEN THE B10 AND THE B10-*x* SUBLINES OF THE C57 BLACK STRAIN OF MICE

Neoplasms	Strains of origin	No. of Mice				Percentage of Susceptibility	
		B10		B10-*x*			
		Susceptible	Resistant	Susceptible	Resistant	B10	B10-*x*
C1498	C57BL/6	259	0	0	612	100	0
B1	"	31	0	0	63	100	0
E 0771	"	69	0	48	65	100	42.4
S653	C57BL/10	41	0	0	67	100	0
B2	"	19	0	0	44	100	0
C954	C57L	63	36	0	112	63.6	0
15091a	A	0	16	0	29	0	0
dBrB	DBA/1	0	50	0	89	0	0
P1534	DBA/3	0	72	3	100	0	2.9
Sarc. 180	?	50	0	33	0	100	100

The following transplantable neoplasms of the mouse were used (Table 1): C1498 myeloid leukemia, B1 and E 0771 (both mammary gland carcinomas), from the subline 6 of the C57BL strain; S653, an undifferentiated rhabdomyosarcoma induced by methylcholanthrene, and the B2 mammary gland carcinoma, from the subline 10 of the C57BL strain; C954 liver carcinoma, from the C57L strain; 15091a and dBrB, both carcinomas of the mammary gland, the former from the A strain, the latter from the DBA/1 stock; P1534, a lymphatic leukemia indigenous to the DBA/3 strain, and Sarcoma 180 which arose in a mouse of unknown source.

The subcutaneous growths were removed, chopped up finely, and inoculated subcutaneously into both axillary regions, by means of a trocar. A total of approximately 78 mg. of tumor tissue was grafted into each mouse, precautions being taken to avoid bacterial contamination of the inoculum. The recipient animals ranged in age

from 5 to 7 weeks at the time of the inoculation and were observed until they died or until the tumors had regressed completely. Records of the pedigrees of the B10 and B10-*x* mice and their hybrids which were inoculated were always kept.

Transplantation Data

The results of the inoculation of several mouse neoplasms into the original subline 10 of C57 black mice (B10) and into the newly developed subline (B10-*x*) are given in Table 1. It is shown that, while four different tumors from the C57BL strain (C1498, B1, S653, and B2) grew progressively in B10 mice, negative results were obtained when these same neoplasms were grafted into animals of the B10-*x* subline. Another tumor from the C57BL strain (E 0771) took in 100 per cent of B10 mice and only in 42.4 per cent of B10-*x* hosts. The C954 tumor from the C57L strain failed to grow in B10-*x* animals but grew progressively in 63.6 per cent of B10 mice. It should be noted that the C57L strain originated by mutation from the C57BR, which was derived from the C57BL before this strain had been inbred. Of the other four alien tumors employed, two of them (15091a and dBrB) failed to grow in either subline. The P1534 leukemia from the DBA/3 strain grew progressively in a small percentage of B10-*x* mice and gave negative results when inoculated into B10 animals. The Sarcoma 180 grew progressively in both B10 and B10-*x* mice.

Considering the manner in which B10 and B10-*x* mice respond to the inoculation of neoplasms indigenous to the C57BL and to the C57L strains, it becomes evident that a fundamental difference exists between these two sublines. This difference is further demonstrated by the results of a complementary experiment in which F_1 hybrids, obtained by crossing B10 or B10-*x* mice to DBA/2 animals, were inoculated either with the C1498 leukemia (from the C57BL strain) or with the P1534 leukemia (from the DBA/3 strain). According to the genetic laws of tumor transplantation (19), if B10 animals were not different from B10-*x* mice the above-mentioned hybrids should be susceptible to both C57BL and to DBA/2 neoplasms (DBA/2 animals are 100 per cent susceptible to the P1534 leukemia from the DBA/3 strain). However, Table 2 shows that, while the grafting of F_1 hybrids from B10 × DBA/2 gave the expected results, the F_1 hybrids from B10-*x* × DBA/2 were susceptible to the P1534 leukemia but resistant to the C1498 leukemia.

Incidentally, 223 F_1 hybrids from B10-*x* × DBA mice were obtained, and no segregation for coat color was observed, all the offspring being solid black.

After it had been shown that the B10 subline was 100 per cent susceptible and the B10-*x* animals 100 per cent resistant to the grafting of the C1498 leukemia, the next step was to investigate whether or not this difference was genetically determined, and, if so, how many genes were involved.

B10 animals were mated to B10-*x* mice, and the F_1, the F_2, and the backcross offspring (obtained by crossing F_1 hybrids to either their B10 or B10-*x* parents) were inoculated with the C1498 leukemia. The data presented in Table 3 show that susceptibility or resistance was inherited according to Mendelian laws, the difference being due to a single gene. Also, the results with the F_1 hybrids obtained by the reciprocal crosses of B10 and B10-*x* animals (♀ B10 × ♂ B10-*x* and ♀ B10-*x* × ♂ B10) rule out any maternal influence as being responsible for the fate of the inoculated leukemic tissue.

DISCUSSION AND CONCLUSIONS

Success or failure in tumor transplantation is conditioned by factors which are shared by both the tumor and the recipient animal. "The fate of the implanted tumor tissue when placed in a given individual (host) is brought about by a reaction between the host, determined to a large extent by its genetic constitution, and the transplanted tumor cell, controlled to some extent by its genetic constitution" (22). The genetic theory of tumor transplantation as put forward by Little and Strong (22) has been confirmed in a large number of publications (1–9, 27). The essential facts which have been established can be summarized as follows:

Susceptibility to tumor grafts is determined by dominant genes[1] which are carried by the animals of the stock in which the tumor arose and is transmitted according to Mendelian laws. The number of hereditary units required for the progressive growth of transplanted tumors may vary with the neoplasms used; some tumors may require just one gene, while some others may depend on the simultaneous presence of eight or more Mendelian factors to grow progressively. A tumor is expected to grow in 100 per cent of the cases when transplanted into inbred animals of the same strain as the donor animal; negative results are generally obtained when it is transferred to an unrelated strain. In this case, temporary growth is frequently observed, which is followed by complete

[1] Incomplete dominance is a possibility that has been pointed out by Dr. P. A. Gorer to Snell (26).

regression after a shorter or a longer period of time. There are tumors which are nonspecific—that is, they can grow progressively in spite of genetic differences between donor and host animals. The offspring from a pair in which one of the parents belongs to a susceptible strain are all susceptible, as is the case with the animals obtained by backcrossing individuals of the F_1 generation to the susceptible parent. The F_2 generation and the backcross of F_1 individuals to the resistant parent show a percentage of susceptible animals varying according to the number of genes required for susceptibility: the larger the number to genes involved, the smaller the percentage of susceptible animals. Little (19) has estimated the percentage of susceptibility in different hybrids from tumor-susceptible and tumor-resistant animals when the simultaneous presence of one or more pairs of genes is required for progressive tumor growth. The genes responsible for the fate of transplanted tumors have been named "histocompatibility genes" (26).

In the vast literature concerning transplantable neoplasms, a few instances can be found in which experiments were designed to investigate whether the offspring from animals which failed to grow a grafted tumor were resistant to the inoculation of the same tumor. Levin and Sittenfield (16) and Morpurgo and Donati (24) performed such experiments with rat sarcomas and found that among the offspring from resistant rats not all the animals were resistant. Similar results were obtained by Cuénot and Mercier (10) in mice. However, as these experiments were done with non-inbred animals, no conclusion can be drawn from the results obtained. MacDowell (23) reports that among two of his inbred strains of mice some of the animals were susceptible, and some survived the inoculation with his line I of leukemia. Contrary to our results, he observed that "by breeding survivors . . . their offspring, when inoculated, gave the same proportion of susceptible animals as in the previous generation." Therefore, in this particular case genetic factors were not responsible for the occurrence of the resistant mice. However, the possibility exists that in other cases a change in the genetic constitution of the original strain of animals might be responsible for an altered reaction between the host and the transplanted tumor.

In our observations, the C57BL/10 strain of mice (B10), which is 100 per cent susceptible to the inoculation of a line of mouse leukemia (C1498), unexpectedly gave rise to animals which proved to be resistant. From these animals, a new subline of mice (B10-*x*) was developed which has been 100 per cent resistant—not only to the C1498 leukemia, but also to three other neoplasms indigenous to the C57BL strain (Table 1). Even with the rather nonspecific tumor (E 0771) from the C57BL strain there occurs a different percentage of progressive growth in B10-*x* mice, than that in the original B10 animals. Also, the 63.6 per cent

TABLE 2

RESULTS OF THE INOCULATION OF THE C1498 LEUKEMIA (FROM THE C57BL/6 STRAIN) AND THE P1534 LEUKEMIA (FROM THE DBA/3 STRAIN) INTO F_1 HYBRIDS OBTAINED BY CROSSING B10 OR B10-*x* WITH DBA/2 MICE

	Grafted with			
	C1498		P1534	
F_1 Hybrids	Susceptible	Resistant	Susceptible	Resistant
♀ B10×♂ DBA/2	10	0	10	0
♀ DBA/2×♂ B10	18	0	22	0
Total	28	0	32	0
♀ B10-*x*×♂ DBA/2	0	40	39	0
♀ DBA/2×♂ B10-*x*	0	19	23	0
Total	0	59	62	0

TABLE 3

RESULTS OF THE INOCULATION OF THE MOUSE LEUKEMIA C1498 INTO HYBRIDS OBTAINED FROM CROSSES BETWEEN B10 AND B10-*x* MICE

	No. of Mice				Expected per cent susceptible if one gene is involved
Hybrids	Inoculated	Susceptible	Resistant	Per cent susceptible	
F_1 (♀ B10×♂ B10-*x*)	108	108	0	100	100
F_1 (♀ B10-*x*×♂ B10)	86	86	0	100	100
Total	194	194	0	100	100
F_2	266	195	71	73.3	75
Backcross (F_1×B10)	121	121	0	100	100
Backcross (F_1×B10-*x*)	216	106	110	49	50

of susceptibility to the C954 tumor from the C57L strain, shown by B10 mice, contrasts with the negative results noted among animals of the B10-*x* subline. We are unable to explain the small percentage of susceptibility (2.9 per cent) to the P1534 leukemia shown by B10-*x* mice, as compared to the complete resistance in the original strain.

Our observations indicate that a fundamental change, interpreted as due to a mutation, occurred in the original subline 10 of the C57 black mice. The new character, resistance to grafted neoplasms from the C57BL strain, has been carried on for seven generations and, as far as the C1498 leukemia is concerned, is inherited according to Mendelian laws (Table 3). Snell (26) has suggested the possibility of mutations being responsible for the occurrence of resistance to tumor grafts among inbred animals of a susceptible strain, by which process new sublines would be developed; our experiments undoubtedly confirm this hypothesis.

The frequency of mutations in inbred strains of animals is pointed out by Haldane (13). The results presented in this paper, and additional ones which will be published later, indicate that some of those mutations may affect the histocompatibility genes and can be easily detected by tumor transplantation. Apparently, it is the first time that this has been demonstrated experimentally and that an isogenic resistant subline of animals, differing from the original strain by a single histocompatibility gene, has been obtained.

SUMMARY

A new subline of mice (B10-*x*) was developed from the C57BL/10 strain (B10) by breeding animals which survived the inoculation with the C1498 leukemia, indigenous to the C57BL/6 strain. Whereas B10 mice are 100 per cent susceptible to this leukemia and to other neoplasms from the C57BL strain, the B10-*x* subline is 100 per cent resistant to the C1498 leukemia and to three out of four other transplantable tumors of the C57BL strain. Susceptibility or resistance to grafts of the C1498 leukemia is inherited according to Mendelian laws, a single gene being responsible for the fate of the inoculated leukemic cells. A mutation occurring in the original, susceptible strain affords the probable explanation for the origin of the isogenic resistant B10-*x* subline. Mutations affecting the histocompatibility genes can be easily detected by tumor transplantations.

REFERENCES

1. Bittner, J. J. A Genetic Study of the Transplantation of Tumors Arising in Hybrid Mice. Am. J. Cancer, **15**:2202–47, 1931.
2. ———. Genetic Studies on the Transplantation of Tumors. IV. Linkage in Tumor 19308A. *Ibid.*, **17**:699–708, 1933.
3. ———. Genetic Studies on the Transplantation of Tumors. V. Tumor 19308B. *Ibid.*, pp. 709–16.
4. ———. Genetic Studies on the Transplantation of Tumors. VI. Tumor 19308C. *Ibid.*, pp. 717–23.
5. ———. Genetic Studies on the Transplantation of Tumors. VII. Comparative Study of Tumors 19308A, B, and C. *Ibid.*, pp. 724–34.
6. ———. Linkage in Transplantable Tumours. J. Genetics, **29**:17–27, 1934.
7. ———. Quadruple Inoculations of an Adeno-Carcinoma. J. Cancer Research, **14**:466–75, 1930.
8. Cloudman, A. M. A Comparative Study of Transplantability of Eight Mammary Gland Tumors Arising in Inbred Mice. Am. J. Cancer, **16**:568–630, 1932.
9. ———. A Genetic Analysis of Dissimilar Carcinomata from the Same Gland of an Individual Mouse. Genetics, **17**: 468–80, 1932.
10. Cuénot, L., and Mercier, L. Études sur le cancer des souris. L'hérédité de la sensibilité à la greffe cancéreuse. Compt. rend. Acad. sc., **150**:1443–46, 1910.
11. Grüneberg, H. Inheritance of Tail Tip Pigmentation in the House Mouse. J. Genetics, **33**:343–45, 1936.
12. ———. The Genetics of the Mouse. Cambridge: at The University Press, 1943.
13. Haldane, J. B. S. The Amount of Heterozygosis To Be Expected in an Approximately Pure Line. J. Genetics, **32**: 375–91, 1936.
14. Heston, W. E. Development of Inbred Strains in the Mouse and Their Use in Cancer Research, pp. 9–31. In: Lectures on Genetics, Cancer, Growth and Social Behavior. Roscoe B. Jackson Mem. Lab., Bar Harbor: Bar Harbor Times, 1949.
15. Law, L. W. Mouse Genetics News No. 2. J. Hered., **39**: 300–308, 1948.
16. Levin, I., and Sittenfield, M. J. Studies in Heredity in Cancer of the White Rat. Proc. N.Y. Path. Soc., **10**:119–26, 1910.
17. Little, C. C. The Genetics of Spontaneous Tumor Formation, pp. 248–78. In: G. D. Snell (ed.), Biology of the Laboratory Mouse. Philadelphia: Blakiston Co., 1941.
18. ———. The Genetics of Spotting. Carnegie Inst. Wash. Yearbook, **23**:42, 1924.
19. ———. The Genetics of Tumor Transplantation, pp. 279–309. In: G. D. Snell (ed.), Biology of the Laboratory Mouse. Philadelphia: Blakiston Co., 1941.
20. Little, C. C., and Bagg, H. J. The Occurrence of Four Inheritable Morphological Variations in Mice and Their Possible Relation to Treatment with X-Rays. J. Exper. Zoöl., **41**:45–91, 1924.
21. Little, C. C.; Murray, W. S.; and Cloudman, A. M. The Genetics of Non-Epithelial Tumor Formation in Mice. Am. J. Cancer, **36**:431–50, 1939.
22. Little, C. C., and Strong, L. C. Genetic Studies on the Transplantation of Two Adenocarcinomata. J. Exper. Zoöl., **41**:93–114, 1924.
23. MacDowell, E. C. Genetic Aspects of Mouse Leukemia. Am. J. Cancer, **26**:85–101, 1936.
24. Morpurgo, B., and Donati, A. Beitrag zur Frage der Vererbung der Anlage zur Geschwulstentwicklung. München. med. Wchnschr., **60**:626–27, 1913.
25. Murray, J. M., and Green, C. V. Inheritance of Ventral Spotting in Mice. Genetics, **18**:481–86, 1933.
26. Snell, G. D. Methods for the Study of Histocompatibility Genes. J. Genetics, **49**:87–103, 1948.
27. Strong, L. C., and Cloudman, A. M. Inbreeding and the Fixation of Genetic Factors Underlying the Transplantability of Cancerous Tissue. Papers, Mich. Acad. Sc. Arts & Letters, **11**:481–83, 1929.

Experimentally Produced Granulosa-Cell Tumors in Rats

B. M. Peckham and R. R. Greene*

(Departments of Obstetrics and Gynecology and Physiology, Northwestern University Medical School, Chicago, Ill.)

Morphology and Hormone Production

In an earlier publication (7) we reported the production of granulosa-cell tumors in rats and rabbits. The method was that used by M. S. and G. R. Biskind (2), in which a portion of one ovary was transplanted to the spleen of a castrated animal, the venous drainage passing through the liver allowing destruction of the produced hormones. The incidence of vascularized adhesions, which permitted the ovarian hormones to enter the general circulation, was reduced by using the intestinal mesentery as the site of transplantation. Under these conditions the hypophyseal gonadotrophin production and action are uninfluenced by the ovarian hormones. A continuous stimulation of the ovarian tissue by gonadotrophins results in the production of granulosa-cell tumors in a high percentage of rats. In this communication the complete data are presented relating to these tumors, their hormone production and their malignancy. The results in the rabbit are less striking and will be presented in another publication.

Results.—Thirty-nine adult, female, albino rats were found free of adhesions at autopsy or had adhesions which were deliberately produced by a secondary operation. The age of transplant at sacrifice varied from 40 to 407 days (Table 1).

All transplants older than 8 months (24) had become definite tumors. Transplants were considered to represent true tumors when they were enlarged, when the architecture was lost, and when no recognizable normal structures (corpora lutea, follicles, ova) remained. Two specimens obtained at 218 days (eighth month) were considered questionable tumors. In the remaining eleven, there were recognizable follicles, corpora lutea, and in some cases even ova, and the architecture remained relatively normal. The tumors both solid and cystic varied between 0.4 and 4.5 cm. in greatest diameter.

Microscopically, the tumors resembled in many respects those of the human granulosa-theca cell group. All the tumors had a definite capsule, grossly appeared highly vascular, and varied in color from blue-black to orange-red. Several were cystic, large cystic spaces being lined by endothelial-like cells, with nodules of tumor tissue present in the wall of the cyst.

Two major cell types could be differentiated in these tumors, the luteinized cell and the unluteinized granulosa cell. At first it was believed that the granulosa cell could be differentiated from a "parenchymal cell" by means of the reticulum stain, the "parenchymal" or so-called interstitial cell of the rat ovary having a network of reticulum fibers about each cell or small groups of two or three cells. However, as more tumors were examined, no distinct difference could be found between the granulosa cells occurring in large groups and those cells which appeared in small groups; furthermore, all gradations in the size of these cell groups could be found in some tumors (Fig. 1). Such a separation was therefore abandoned. The granulosa cells usually have scant cytoplasm, are often closely packed, and are devoid of recognizable cell membranes. The nuclei appear small, round, and are often slightly irregular and hyperchromatic. Mitoses are common but are most frequent in the larger groups of these cells.

The luteinized cells usually have an individual reticular network. However in two of the tumors, (Nos. 43R1, 21R1) there were large groups of lutein cells which were devoid of reticulum (Fig. 2). These cells contain a large amount of lightly eosinophilic homogenous or granular cytoplasm. The cell membrane is clearly visible. The nuclei are large, vesicular, and contain one or two nucleoli. Mitoses were noted in these cells in one tumor (No. 46R1).

This investigation sheds no light on the origin of these cells, but this problem seems of little importance, as their primary origin from a single cell type can hardly be questioned.

The tumors were made up of granulosa cells and luteinized cells in varying proportions. In some the granulosa cells were present in small cords and tubules, the predominant cell being the luteinized cell. One such tumor (Fig. 3*a* and *b*) was made up

* Financed in part by a grant from Ciba Pharmaceutical Co. and in part by a cancer control grant from the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

Received for publication September 11, 1951.

almost entirely of nodules of luteinized cells, arranged in cords separated by sinusoids filled with blood. However, granulosa cells were present at the periphery of these nodules, albeit in small numbers. At the other extreme were tumors which consisted almost entirely of masses of unluteinized, granulosa cells (Fig. 4). In some there were masses of granulosa cells surrounded by small cavities filled with blood or serous fluid. One of the tumors contained areas of spindle cells resembling the human thecal cells (Fig. 5) and another, areas of cells with a sarcomatous appearance (Fig. 6).

Malignancy.—Metastases were present in the kidney and liver of the animal (No. 21R1) bearing the tumor with sarcoma-like areas (Figs. 7, 8). However, this animal was one of four in which the capsule of the tumor was opened surgically and sutured to the anterior abdominal wall 10 days prior to sacrifice. It was hoped thereby to divert the produced hormones into the general circulation. Of course it is possible that vascular metastasis followed this procedure; however, from the gross size of the kidney lesion and the associated marked atrophy of kidney parenchyma, it seems more likely that the lesion had been present for more than 10 days.

Discussion.—The scattered distribution in the age of the transplants makes conclusions as to pathogenesis impossible. Biskind and Biskind (1) have described progressive luteinization of the transplants and the formation of luteomas from nodules of lutein cells which grow displacing normal structures. We have not observed such nodules. The two questionable tumors (218 days of age) showed a loss of normal architecture and an encroachment by cells similar to granulosa cells in cords and small groups at the periphery of corpora lutea. Transplants of lesser age did not differ materially from the normal rat ovary except for the large number of corpora lutea and, as the age increased, a gradual reduction in the number of follicles and ova.

Hormone production.—In the rat the vagina, uterus, and prostate are sensitive indicators of the presence of hormones in the general circulation. After castration and ovarian transplantation to the portal circulation daily vaginal smears taken prior to tumor formation (265 days) showed no evidence of stimulation (see Table 2 for details). Animals sacrificed during this period showed no evidence, either gross or microscopic, of vaginal or uterine stimulation. Apparently, the liver was able to inactivate totally any hormone produced by the transplant.

Of the 24 rats developing tumors, 22 were studied by daily vaginal smears for 1–2 weeks between the 278th and 357th day after the transplantation. Three showed evidence of intermittent or constant estrogenic stimulation (Table 2). Smears were taken from thirteen of these 22 rats daily for from 1–3 weeks between the 339th and 407th day (the other nine having been sacrificed previously). Five of these thirteen showed evidence of constant or intermittent estrogenic stimulation, indicating that the liver in these rats was unable completely to inactivate produced estrogens. In no case were there regular cycles.

Prostatic tissue from immature male rats was implanted subcutaneously in ten of these thirteen rats from which smears were taken a second time. This tissue took in seven, and, of these, three showed a response suggestive of androgenic stimulation. However, it is known (4, 5) that progesterone in large doses will produce similar prostatic changes.

TABLE 1

AGE DISTRIBUTION OF TRANSPLANTS AT SACRIFICE*

Transplant age (months)	1	2	3	4	5	6	7	8	9	10	11	12	13	14
No. animals	0	3	6	1	0	0	0	2?	*1*	*2*	*5*	*7*	*5*	*4*

* Italicized figures represent true tumors.

At sacrifice the vaginas of all rats bearing tumors showed evidence of stimulation.[1] The uteri did not show evidence of stimulation until the tumors were 328 days or older. After this age all examined were stimulated (in two cases the uteri were not available). Four types of vaginal stimulation were seen: mucification of variable degree, proestrus, estrus, and diestrus.

Proestrous and estrous reactions suggest estrogen production without measurable amounts of progestational or androgenic hormones. Mucification on the other hand suggests that one or the other or both of these hormones were present. The diestrous reaction suggests estrogen production insufficient to produce full estrus. These vaginas were not atrophic.

No attempt was made to classify the uterine response. Signs of active growth such as mitoses in the lining epithelium with increased uterine size

[1] The vaginas of three rats were not available. The uterus of one of these was highly stimulated, suggesting that vaginal stimulation of this animal was likely.

(beyond that seen in the castrate) were considered evidence of stimulation.

Summary and conclusion.—

1. Ovarian tissue was transplanted to the spleen or intestinal mesentery in castrate rats. The transplants of all rats sacrificed 8 or more months later (24) had become granulosa-cell tumors.

2. The morphology of these tumors is discussed, and the metastases of one tumor are described.

3. Evidence is presented that estrogens are produced in large quantities and that androgens and/or progestational hormones are also produced in detectable quantities.

TRANSPLANTABILITY

Five tumors were chosen for transfer to male and female albino rats 60–90 days of age. A portion of the tumor obtained at operation or sacrifice was minced with scissors in physiological saline and injected through a 16-gauge hypodermic needle subcutaneously into intact and castrate animals and intrasplenically into castrates. The 76 surviving rats were sacrificed from 311 to 346 days later.[2] The secondary tumors were studied histologically, and the uteri, vagina, or prostate of each

[2] Unfortunately some 50 or 60 rats in this series were accidentally destroyed.

TABLE 2

DETAILED DATA ON RAT TUMORS

Animal no.	Transplant age at sacrifice	Greatest diameter (cm.)	Condition of			Vaginal smear results					
			Vagina	Uterus	Prostate	1st Occasion			2d Occasion		
30R*	218	0.2	Atrophic	Atrophic							
35R*	218	0.4	"	"							
32R	265	1.0 (cystic)	Low Mucified	"							
40R1	288	4.5 (cystic)	"	"							
51R1	295	1.3	"	"		287	Constant diestrus	294†			
39R1	304	1.2	Diestrus Stimulated	"		278	"	291			
41R1	304	1.2	Mucified	"		278	"	291			
52R1	304	0.8	Not available	Not available		278	"	291			
33R1	328	1.5	Proestrus	Stimulated		280	"	293			
46R1	329	1.5	Mucified	"		280	"	293			
4R1	352	2.2	Proestrus	"		344	Constant estrus	352			
37R1‡	354	1.0	Mucified	"	no take	301	Constant diestrus	514	339	Constant diestrus	354
7R1	356	1.2	Not available	Not available		344	"	356			
38R1‡	357	2.6	Diestrus Stimulated	Stimulated	Atrophic	301	"	314	339	Constant diestrus	357
43R1	358	1.4	Proestrus	"		302	"	315	349	Constant estrus	358
47R1	358	3.0	Proestrus	"		302	Intermittent estrus and diestrus	315†	340	Proestrus and estrus. Occas. diestrus	358
42R1§	359	1.6	Not available	"	No take	302	Constant diestrus	315	340	Intermittent estrus and diestrus	359
50R1	361	1.5	Marked mucification	"	No take	302	"	315	340	Constant diestrus	361
56R1	361	1.8	Proestrus	"	Atrophic	302	"	315	340	Intermittent proestrus	361
35R1	364	1.7	High mucified	"	Stimulated	287	Intermittent estrus and diestrus	300	339	Constant diestrus	364
29R1‡	367	0.4	Estrus	"	Stimulated	291	Constant diestrus	304	343	Diestrus prior to op.; estrus post-op.	367
9R1	387	0.6	Slight mucification	"		344	"	357			
14R1	399	1.6	Mucified	"		330	"	343	382	Constant diestrus	399
20R1	402	1.4	"	"	Atrophic	330	"	343	382	"	402
21R1‡	403	2.5	High mucified	"	Stimulated	330	"	343	382	"	403
13R1	407	2.6	"	"	Atrophic	330	"	343	382	"	407

* Questionable tumors.
† Days smeared inclusive.
‡ Animals in which tumor was sutured to anterior abdominal wall.
§ Adhesion between tumor and one uterine horn.

rat was examined to determine its response to produced hormones. Granulosa cells predominated in three and the luteinized cells in two of the original transplanted tumors.

Results.—Subcutaneous transplantation of all 5 tumors to castrate rats was successful. Of 33 rats (20♂, 13♀) subcutaneous tumors were found in 14 (10♂, 4♀). Intrasplenic transplantation was less successful; of 18 rats (9♂, 9♀) available for study, only 2 females had developed tumors, both having received transplants from tumor No. 18R1. Subcutaneous transplantation of all tumors into intact rats (7♂, 18♀) was totally unsuccessful.

Morphology.—The secondary tumors were single and small (approximately 2–6 mm. in diameter) with the exception of those in one rat which developed multiple subcutaneous tumors derived from the primary tumor No. 21R1. These tumors had an extremely fibrous consistency, measured from 1 to 2 cm. in diameter, and were microscopically identical to the sarcoma-like areas of the parent tumor (Fig. 6). All (large and small) were apparently encapsulated except the two intrasplenic tumors, though these were sharply demarcated from the normal splenic tissue. The smaller tumors appeared highly vascular.

The unluteinized granulosa cells and luteinized cells seen in the primary tumors made up almost all these tumors, except for the sarcomatous appearance of No. 21R1. The original tumor from this animal also contained areas of normal appearing granulosa and luteinized cells as well, which explains their presence in the other transplants from this tumor (Fig. 13*a* and *b*).

The arrangement of the cells varied considerably from the parent tumor, with very few exceptions. Furthermore, transplants from the same tumor in different animals often did not resemble one another (Figs. 10–12). The sex of the recipients apparently did not influence the microscopic appearance of the tumors.

Hormone production.—All six female rats bearing tumor transplants (from tumors Nos. 18R1, 21R1, 35R1) showed marked vaginal cornification (Fig. 12*c*). The uteri of five showed squamous metaplasia (Figs. 12*b* and 13*c*), a sign of long continued estrogenic stimulation. In one the uteri were in an estrous condition. Of the ten male rats bearing transplants, four showed prostatic stimulation. In one it was marked (Fig. 9*c*). Stimulation was noted only in rats bearing transplants from tumors No. 46R1 and No. 21R1 (Figs. 9*a* and 6). It is interesting that the prostatic transplants in the latter animal bearing the original tumor also showed stimulation (Table 2). However, two other male rats bearing transplants of tumor 46R1 and one with transplants from 21R1 had atrophic prostate glands.

The microscopic appearance of the transplants in animals showing prostatic stimulation did not differ notably from those not showing this stimulation.

Discussion.—Granulosa-cell tumors induced by intrasplenic ovarian implantation in mice have been carried through subpassages by intrasplenic implantation in castrate mice (3). Such tumors have occasionally metastasized either before or after intrasplenic subpassage (3, 6). Once malignant properties have appeared, subcutaneous transplantation into intact mice has been possible.

In the rat, subpassage of these tumors apparently has not been attempted, and metastasizing tumors have not been reported. The apparently malignant tumor in our series (No. 21R1) was not autonomous, as on subpassage no takes occurred in intact rats. Subcutaneous subpassage of this tumor in one castrate, however, did produce multiple tumors (microscopically of sarcomatous appearance) which grew to much larger proportions than did other transplants from the same tumor or other tumors.

One would anticipate that intrasplenic subpassage would give rise to larger tumors, assuming that the liver would destroy produced hormones until the tumor reached the approximate size of the parent tumor. This was not true of the two intrasplenic transplants, both being approximately 6 mm. in diameter (both of these tumors were free of adhesions). However, despite the small size of these transplants both animals showed evidence of circulating ovarian hormones (uterine and vaginal stimulation). It may be that the hormone production was much greater than the size of the transplant would indicate or that the type of hormone produced was not inactivated by the liver.

Summary and conclusions.—

1. Subpassage was successful from five of the original granulosa-cell tumors in castrate male and female rats.

2. These secondary tumors were not fully autonomous, as no growth was obtained in the intact rat.

3. All (six) females bearing transplants showed estrogenic stimulation of the uterus and vagina.

4. Of ten males bearing transplants, four showed stimulation of the prostate, suggesting androgenic or progestational stimulation.

5. The sex of the recipient apparently did not influence the microscopic appearance of the tumors.

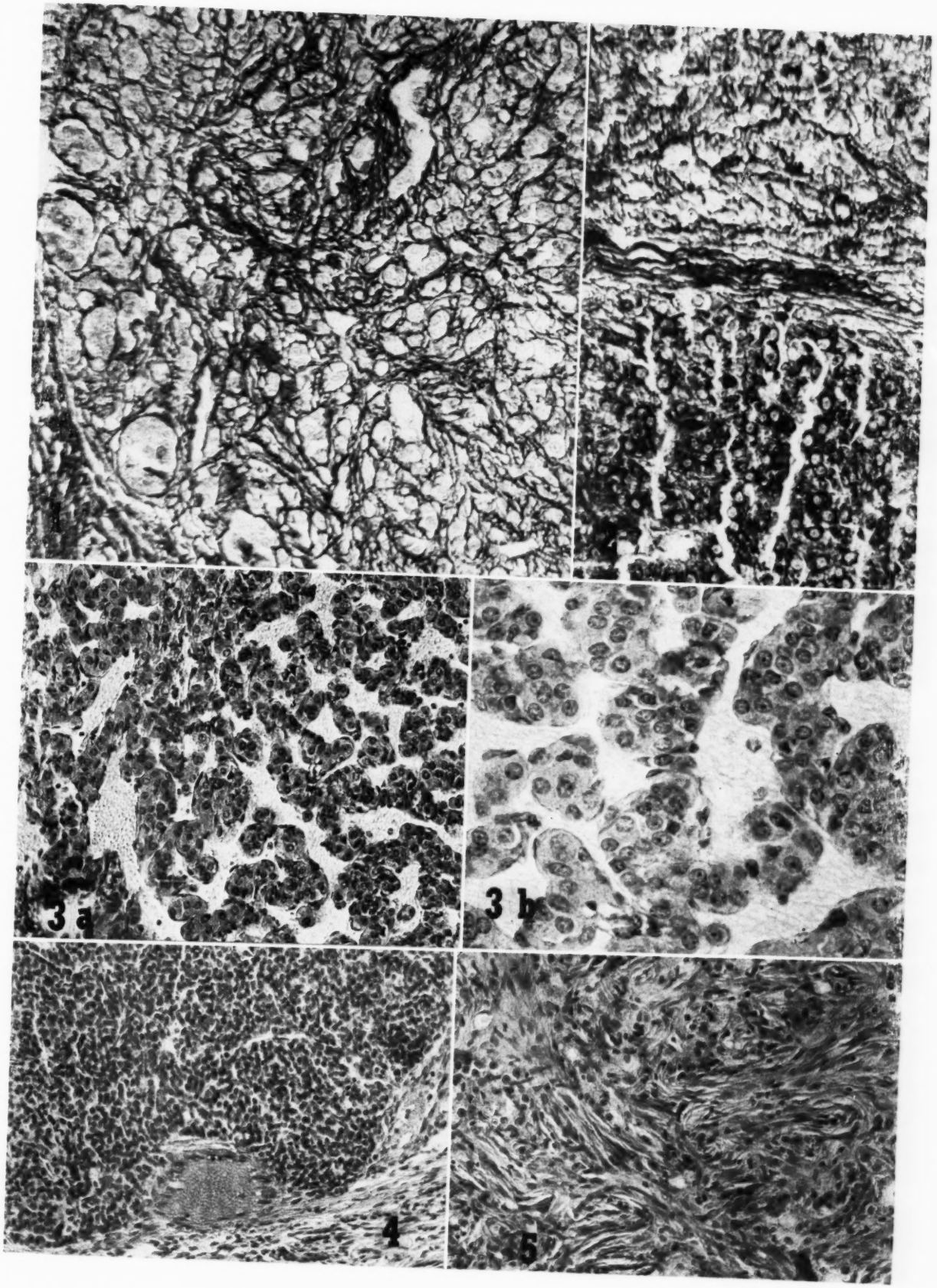

3 a
3 b
4
5

FIG. 1.—Tumor No. 43R1. Reticulum stain demonstrating reticulum surrounding individual and groups of unluteinized granulosa cells. ×250.

FIG. 2.—Tumor No. 43R1. Reticulum stain. The luteinized cells in the lower half of the picture are devoid of reticulum. The cytoplasm of these cells takes a deep brown stain. ×250.

FIG. 3*a*.—Tumor No. 51R1. Hematoxylin and eosin stain. Cords of luteinized cells separated by sinusoids filled with blood. There were small groups of unluteinized granulosa cells (see Fig. 4) at the periphery of these nodules. ×250.

FIG. 3*b*.—Tumor No. 51R1. ×800.

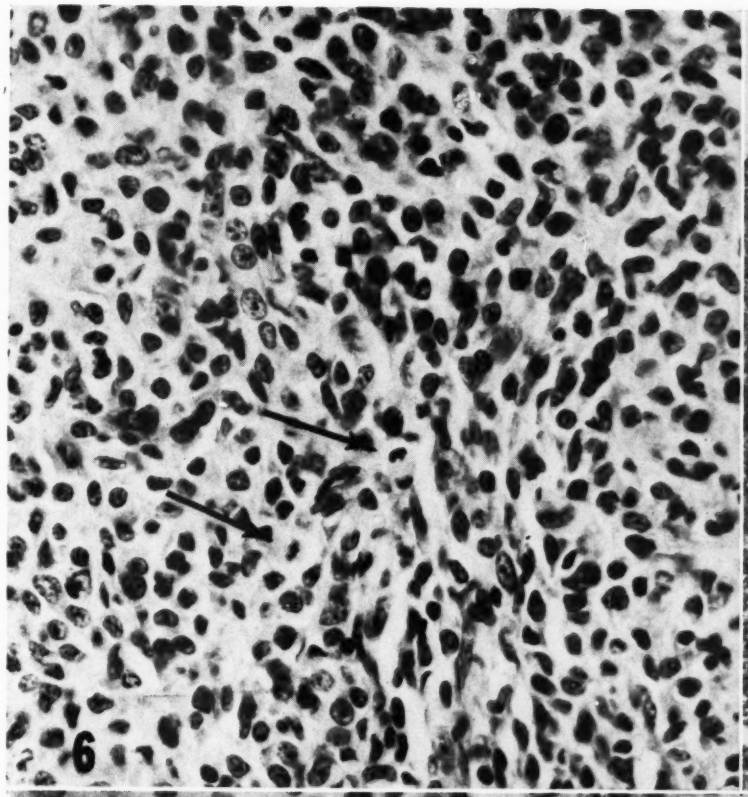
FIG. 4.—Tumor No. 38R1. An almost pure granulosa-cell tumor showing a portion of the fibrous capsule. ×75.

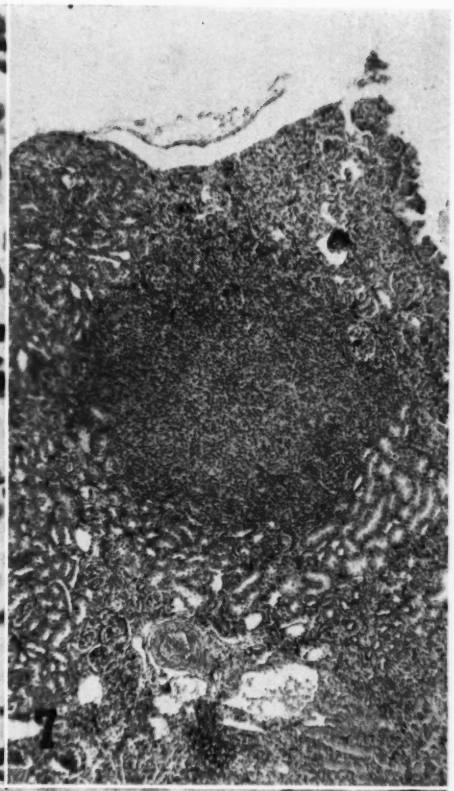
FIG. 5.—Tumor No. 18R1. Area suggestive of human theca-cell tumor. Other areas in this tumor were similar to No. 46R1 (Fig. 9*a*). ×225.

FIG. 6.—Tumor No. 21R1. Sarcomatous area. This tumor metastasized to the kidney and liver (Figs. 7, 8). Numerous mitotic figures were present (arrow). ×800.

FIG. 7.—Tumor No. 21R1. Metastic nodule in an atrophic kidney (see text). ×40.

FIG. 8.—Tumor No. 21R1. Metastic tumor tissue (left) invading and destroying liver tissue (right). ×900.





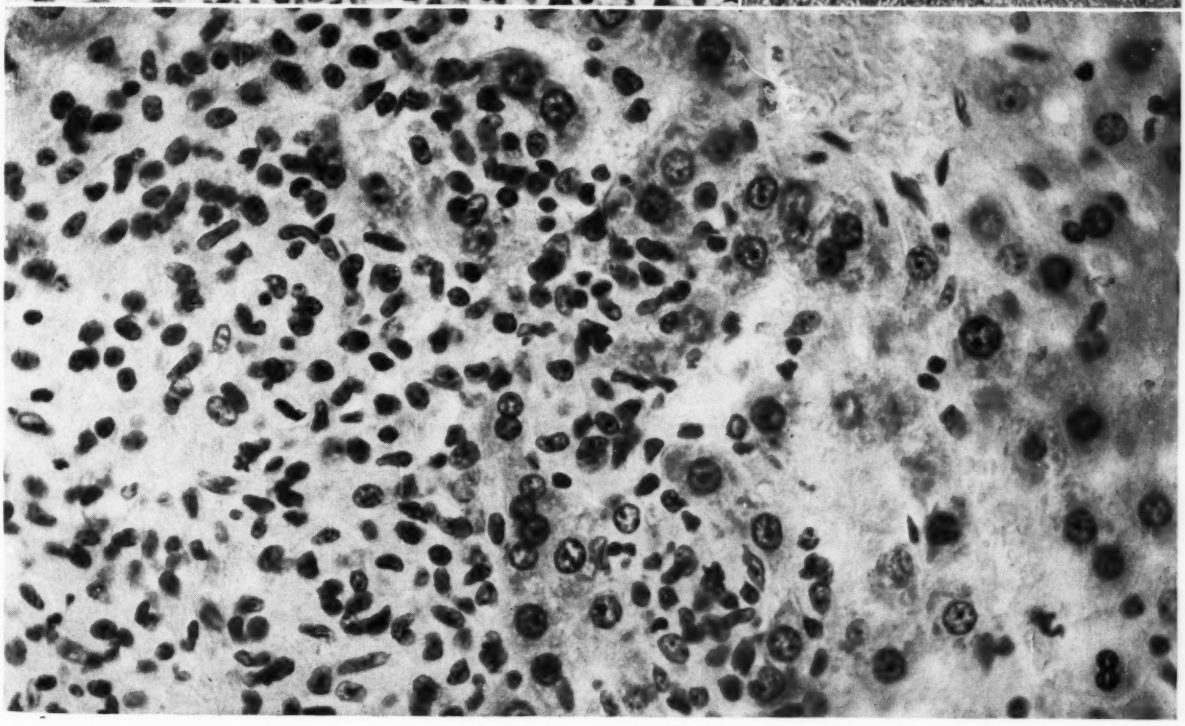


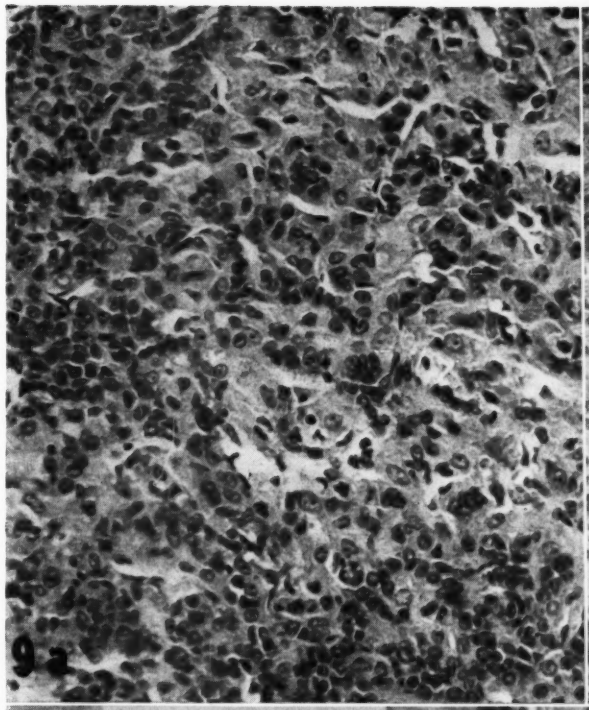
FIG. 9*a*.—Tumor No. 46R1. Parent tumor, a portion of which gave rise to the tumor pictured in Figure 9*b*. ×325.

FIG. 9*b*.—Tumor No. 46R1 ♂ 11. Subcutaneous subpassage in a castrate male rat from tumor depicted in Fig. 9*a*. Note masses of unluteinized granulosa cells, luteinized cells, and human theca-like cells. The prostate from this rat showing androgenic stimulation appears in Figure 9*c*. ×325.

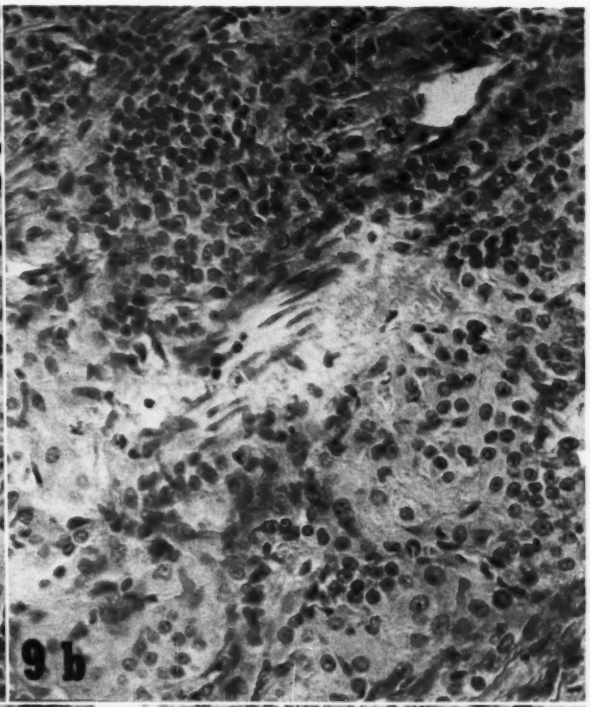
FIG. 9*c*.—Prostate from No. 46R1 ♂ 11. Evidence of androgenic stimulation (light areas in the glandular epithelium at arrow) presumably the result of hormone production by the tumor (Fig. 9*b*) in this castrate rat. ×1,000.

FIG. 10.—Tumor No. 18R1 ♀ 1. Subcutaneous subpassage in a castrate female from the tumor shown in Figure 9*b*. Note the striking difference in microscopic appearance from the original tumor and from the tumors in Figures 11 and 12*a*. All three (in castrate females) were derived from the same tumor. ×325.

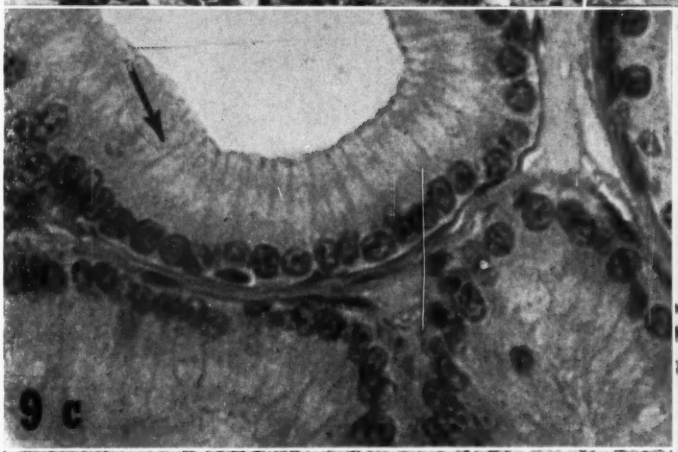
FIG. 11.—Tumor No. 18R1 ♀ 3. See legend for Figure 10. ×325.



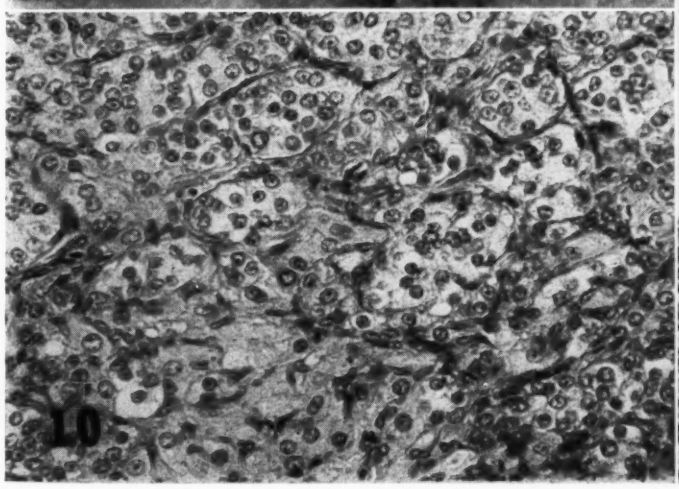
9a



9b



9c



10

11

FIG. 12*a*.—Tumor No. 18R1 ♀ 5. See legend for Figure 10. ×325.

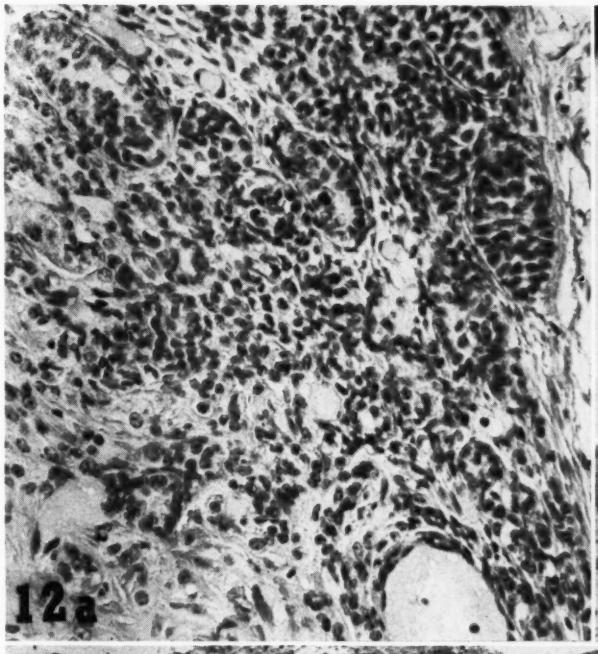
FIG. 12*b*—Uterus from No. 18R1 ♀ 5. Squamous metaplasia of the uterine epithelium in a castrate female. The tumor (Fig. 12*a*) is the probable source of estrogenic stimulation. ×20.

FIG. 12*c*.—Vaginal mucosa from 18R1 ♀ 5. Cornification suggesting high level estrogenic stimulation. ×100.

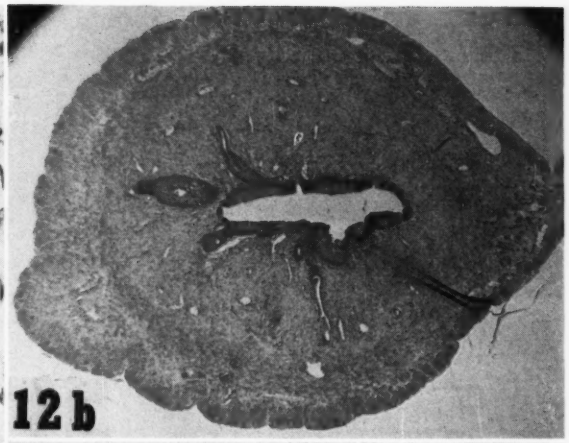
FIG. 13*a*.—Tumor No. 21R1 ♀ 8. Subcutaneous subpassage in a castrate female from tumor shown in Figure 6. Note sinuses filled with blood cells, light areas consisting of partially luteinized cells, and darker areas of unluteinized granulosa cells. ×100.

FIG. 13*b*.—Tumor No. 21R1 ♀ 8. Higher magnification to show unluteinized and luteinized cells in greater detail. ×175.

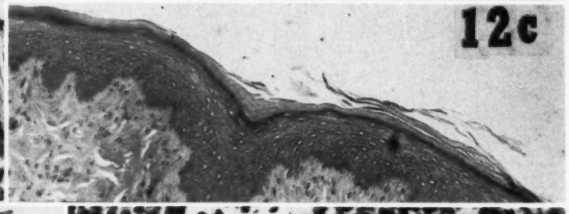
FIG. 13*c*.—Uterus from 21R1 ♀ 8. Metaplasia of uterine epithelium, a sign of estrogenic stimulation in the animal bearing the tumor in Figure 13*a*. ×100.



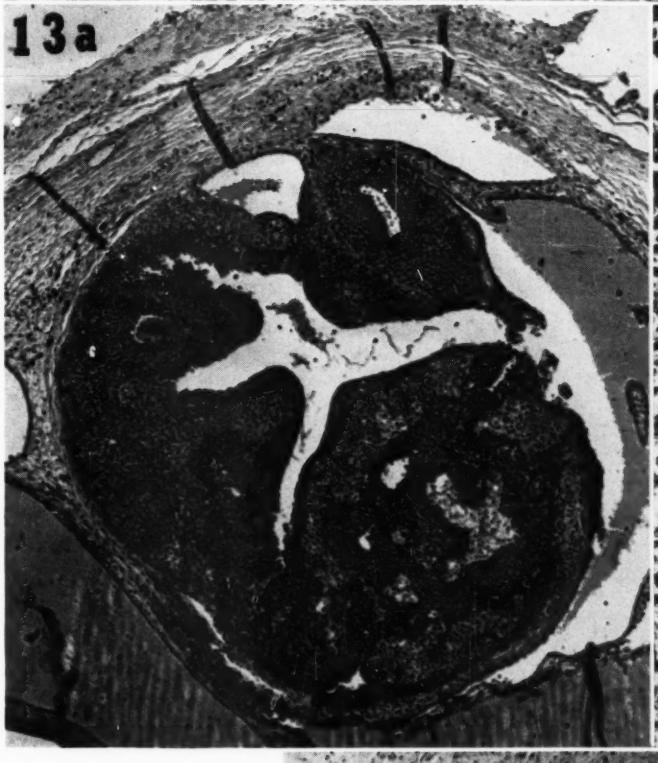
12a



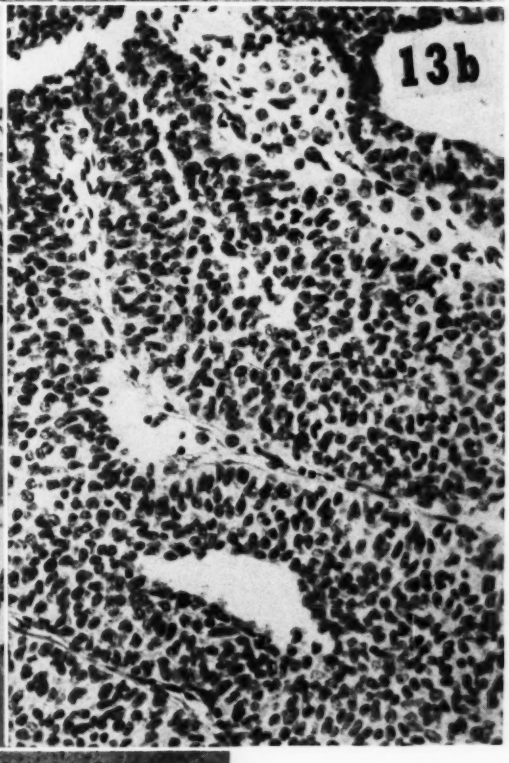
12b



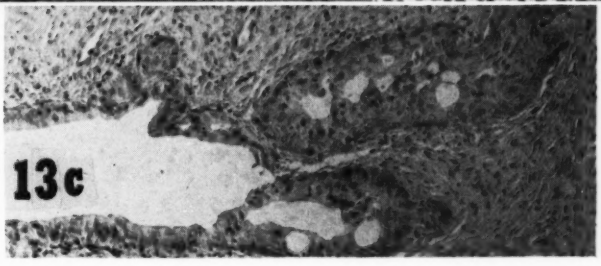
12c



13a



13b



13c

REFERENCES

1. BISKIND, G. R., and BISKIND, M. S. Experimental Ovarian Tumors in Rats. Am. J. Clin. Path., **19**:501–21, 1949.
2. BISKIND, M. S., and BISKIND, G. R. Development of Tumors in the Rat Ovary after Transplantation into the Spleen. Proc. Soc. Exper. Biol. & Med., **55**:176–79, 1944.
3. FURTH, J., and SOBEL, H. The Neoplastic Transformation of Granulosa Cells in Grafts of Normal Ovaries into Spleens of Gonadectomized Mice. Cancer Research, **7**:710, 1947.
4. GREENE, R. R.; BURRILL, M. W.; and IVY, A. C. Progesterone is Androgenic. Endocrinology, **24**:351–57, 1939.
5. GREENE, R. R.; BURRILL, M. W.; and THOMSON, D. Further Studies on the Androgenicity of Progesterone. Endocrinology, **27**:469–72, 1940.
6. LI, M. H. Malignant Granulosa Cell Tumor in an Intrasplenic Ovarian Graft in a Castrate Male Mouse. Am. J. Obst. & Gynec., **55**:316–20, 1948.
7. PECKHAM, B. M.; GREENE, R. R.; and JEFFRIES, M. E. Granulosa Cell Tumors in Female Rats and Rabbits. Science, **107**:319–20, 1948.

The Identification of Aromatic Polycyclic Hydrocarbons in Carbon Blacks*†

HANS L. FALK and PAUL E. STEINER

(*Department of Pathology, University of Chicago. Chicago 37, Ill.*)

In previously reported experiments, the carcinogenic potency for mice of extracts of processed rubber was traced to the presence of 3,4-benzpyrene and related compounds (13). A search for the origin of these hydrocarbons was next undertaken. Samples of crude rubber, both natural and synthetic, did not contain any of these hydrocarbons when they were sought by extraction, chromatography, and ultraviolet spectrophotometry.[1] A study of a typical formula for the processing of rubber suggested carbon black as the most likely source of the hydrocarbons. Samples of 24 carbon blacks were then examined by methods similar to those mentioned above. The absorption spectra of 3,4-benzpyrene and six related aromatic hydrocarbons were detected in a great number of the carbon blacks. The aromatic hydrocarbons found in carbon blacks were the same as those identified in processed rubber except that (*a*) coronene was found in carbon blacks but not in rubber and (*b*) chrysene and the phenanthrene derivative, which had been detected in processed rubber, were not found in any carbon black.

Extensive literature exists on the possible relationship of soot, carbon black, and related dusts to human skin and respiratory tract cancer, on the carcinogenicity of soot and dusts in experimental animals, on the carcinogenicity of extracts from such atmospheric contaminants, and on the physicochemical identification of carcinogens in such products. These subjects will be considered in detail in the discussion, because they are closely related to the work here reported and to follow. Both soot and carbon black are forms of amorphous carbon made by the pyrolysis of organic substances. Soot is the more general term; it includes all carbon blacks. Carbon black is a commercial product made by the controlled combustion of natural gas and related fuels. Its content of acetone-extractable tars is usually below 1 per cent. In some soots this figure may go much higher.

The present paper deals with the identification of polycyclic aromatic hydrocarbons in different types of carbon blacks and with their possible significance. The following paper is concerned with the adsorptive capacity of carbon black for chemical carcinogens. A subsequent paper is planned to deal with the biological significance of these observations to carcinogenesis.

EXPERIMENTAL

Twenty-four samples of carbon blacks were obtained from four different manufacturers. One-pint aliquots of the blacks were placed in large flasks, enough redistilled benzene was added to cover the material, and the suspension was heated on a hot-water bath under a reflex condenser for 30 minutes. When cool, the mixture was filtered, and the residue was re-extracted. The combined filtrates were taken to dryness on a hot-water bath under vacuum. The residue was dissolved in redistilled petroleum ether and chromatographed on a column of activated alumina. The results of a typical chromatogram are shown in Table 1. The individual fractions obtained by chromatography were taken to dryness under a stream of nitrogen and were analyzed for their absorption spectra in a Beckman ultraviolet spectrophotometer with ether as the solvent. To resolve mixtures of hydrocarbons, extraction with sulfuric acid was used to isolate 3,4-benzpyrene (3), and anthanthrene was purified by means of precipitation with iodine (4). For identification of the compounds by their absorption spectra, what were believed to be the corresponding purified commercial aromatic hydrocarbons were analyzed spectrophotometrically, and the spectra were compared. The absorption spectra of compounds for which commercial prod-

* This work was supported by a research grant from the Committee on Growth acting for the American Cancer Society, and by an institutional grant from the American Cancer Society.

† A preliminary report of this work was presented before the American Association for Cancer Research, April 27–29, 1951, and was published in abstract form in the Scientific Proceedings of that meeting.

[1] H. L. Falk, unpublished data.

Received for publication September 17, 1951.

ucts were not available for comparison were identified by comparison with data given by Clar (9, 10).

The 24 samples included 7 commercial brands of channel blacks and 17 brands of furnace blacks. The aromatic hydrocarbons identified in these blacks are given in Table 2. No aromatic hydrocarbons could be eluted from the channel blacks or from a few of the furnace blacks. In most of the furnace blacks, however, the following seven polycyclic aromatic hydrocarbons were identified: pyrene, fluoranthene, 3,4-benzpyrene, anthanthrene, 1,2-benzpyrene, 1,12-benzperylene, and coronene. The structural formulae of these compounds are given in Chart 1. One absorption spectrum has not been identified as yet. The absorption spectra of the eight compounds are given in Charts 2 to 9, together with spectra of commercial samples of these compounds. The identification was confirmed by melting-point determinations in the cases of pyrene, fluoranthene, 1,12-benzperylene, and coronene. Not enough purified crystals of the remaining three compounds were obtained for confirmatory tests. In a furnace black of a type not investigated in this study pyrene had been previously isolated by Campbell *et al.* (6), and fluoranthene had been isolated by Rehner (35).

It is of interest to note that all the compounds detected in this study belong to the series of aromatic hydrocarbons with peri-condensed ring systems, i.e., one or more carbon atoms are shared by three rings. This is the class of hydrocarbons which has the smallest proportion of hydrogen to carbon for their molecular weight.

The carbon blacks which were studied were obtained from commercial sources. We did not verify the nature of the materials from which they were manufactured; when it is available, information on the raw material is given in Table 2. According to method of manufacture all samples were stated to be either channel blacks or furnace blacks. All channel blacks were made from natural gas, while the furnace blacks were made from natural gas, gasoline fractions, or petroleum residues. In the channel blacks no aromatic hydrocarbons could be detected. Many of the furnace blacks, however, regardless of the raw material used, contained all

TABLE 1

CHROMATOGRAM OF A SEMI-REINFORCING FURNACE BLACK

Fractions	Eluant: per cent ether in petroleum ether	Sulfuric acid treatment	Physical properties	Chemical nature
1– 7	0–12			
8–10	14–18		Colorless crystals	Pyrene
11–12	20–22		Yellow crystals	Fluoranthene
13	24		Yellow crystals	Unknown
14	26	Soluble	Yellow oil	3,4-Benzpyrene
		Insoluble	Yellow crystals	1,2-Benzpyrene
15	28	Soluble	Orange crystals	Anthanthrene* and 1,12-Benzperylene
		Insoluble	Yellow crystals	1,12-Benzperylene
16	50	Soluble	Orange crystals	Anthanthrene
		Insoluble	Orange crystals	Coronene

* Further purification by iodine precipitation.

TABLE 2

POLYCYCLIC AROMATIC HYDROCARBONS IDENTIFIED IN CARBON BLACKS

Classification of carbon blacks	Starting material given by manufacturer	Average particle diameter in mμ	Compounds identified*
Channel blacks:			
1. High color	gas	10	None
2. " "	gas	14	None
3. " "	gas	17	None
4. Ink	gas	25	None
5. Medium processing	gas	29	None
6. Easy processing	gas	29	None
7. " "	gas	30	None
Furnace Blacks:			
8. High abrasion	oil	26	1,2
9. " "	gas and gasoline	35	1,2 (3–6†)
10. Reinforcing		38	None
11. "		39	1†
12. "		39	1†
13. High structure	oil	41	1,2
14. Fine		43	1–7
15. High modulus	gas and gasoline	51	1,2 (3–6†)
16. " "		51	1,2
17. " "		60	1,2 (3–6†)
18. Semi-reinforcing non-staining		75	1,2 (3–6†)
19. Semi-reinforcing	gas	80	1–7
20. "	gas	80	1–7
21. "	oil	?	1–7
22. "		83	1–7
23. High modulus		95	1–7
28. Semi-reinforcing		160	1–7‡

* 1 = Pyrene; 2 = fluoranthene; 3 = 1,2-benzpyrene; 4 = 3,4-benzpyrene; 5 = 1,12-benzperylene; 6 = anthanthrene; 7 = coronene.

† Traces only.

‡ Larger than average yield.

seven of the aromatic hydrocarbons; the remainder contained few or none (see Table 2). The possibility exists that the aromatic hydrocarbons found in many of the furnace blacks might have been introduced in a preformed state with the crude starting material. This possibility could be eliminated as the sole explanation, if it could be shown that the same aromatic hydrocarbons are present in carbon black made from gas in the laboratory, since these aromatic hydrocarbons are solids and are not volatile at room temperature.

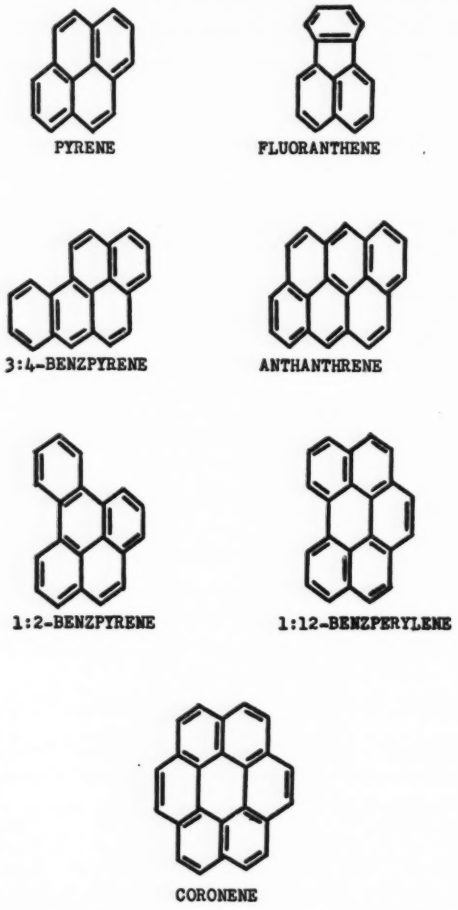


CHART 1.—Structural formulae of compounds detected in furnace blacks.

Accordingly, gas of known composition, which was stated to be a mixture of natural and coke oven gas, was burned in the laboratory on a Bunsen burner with a supply of air insufficient for complete combustion, and the flame was made to impinge on an inverted metal tray. The soot so formed was extracted with benzene. The fluorescent extract was chromatographed. Spectrophotometric analysis revealed the presence of the same seven aromatic hydrocarbons found in many furnace blacks. This observation establishes the fact that these aromatic hydrocarbons can be formed from gas in the process of carbon black formation.

A correlation can be made between the physical properties of the carbon particles of the blacks with the extraction of aromatic polycyclic hydrocarbons. In 11 out of 24 samples studied, 3,4-benzpyrene was identified by its spectrum. In addition, six other closely related aromatic hydrocarbons were detected. Whenever 3,4-benzpyrene was found, most of the others were also present. These eleven carbon blacks belong to the groups of "high modulus furnace" and "semi-reinforcing furnace" blacks which are said to have an average particle diameter of 43–160 mμ. Only two hydrocarbons could be obtained from a number of furnace blacks (Table 2); they belong to the "high abrasion furnace" and "reinforcing furnace" blacks. They have an average particle diameter of 26–50 mμ. The third group, from which aromatic hydrocarbons could not be eluted in detectable amounts, is composed of the "channel blacks" with particle size ranging from 10 to 30 mμ.

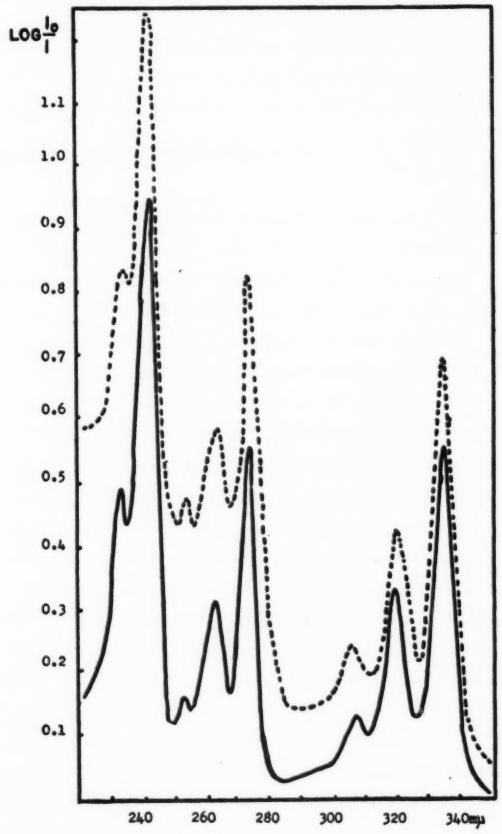


CHART 2: ——— Absorption spectrum of purified commercial pyrene.
-------- Absorption spectrum of pyrene isolated from carbon black.

No quantitative experiments were made, because, as will be shown in the following paper, the phenomenon of adsorption makes quantitative re-

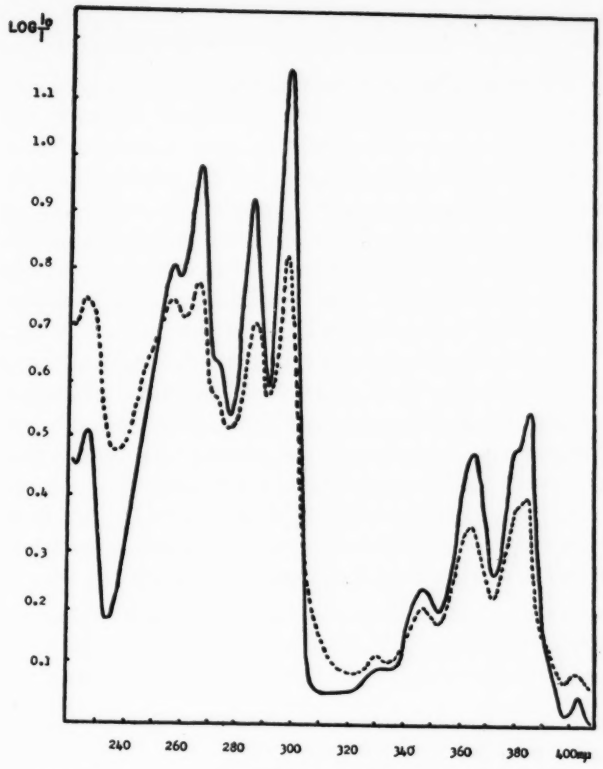


CHART 3: ——— Absorption spectrum of purified commercial 3,4-benzpyrene.
-------- Absorption spectrum of 3,4-benzpyrene ioslated from carbon black.

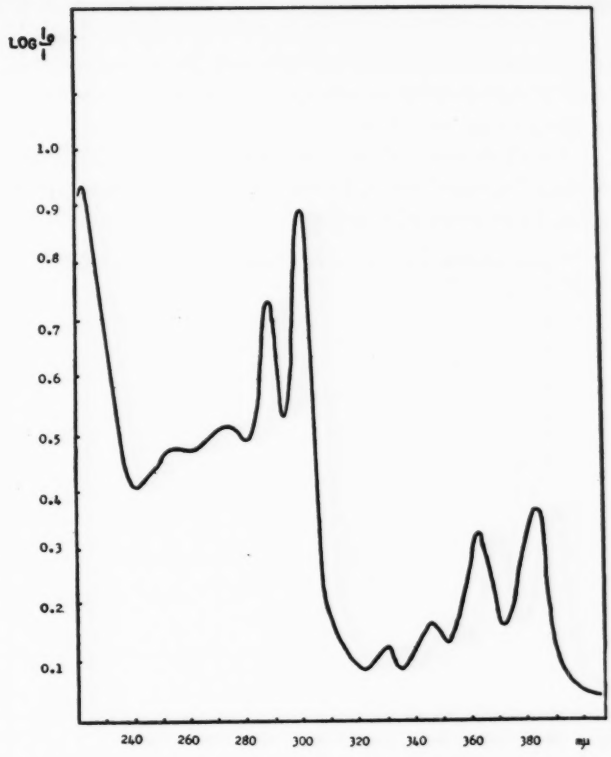


CHART 5.—Absorption spectrum of 1,12-benzperylene isolated from carbon black. (Compare with Clar [9].)

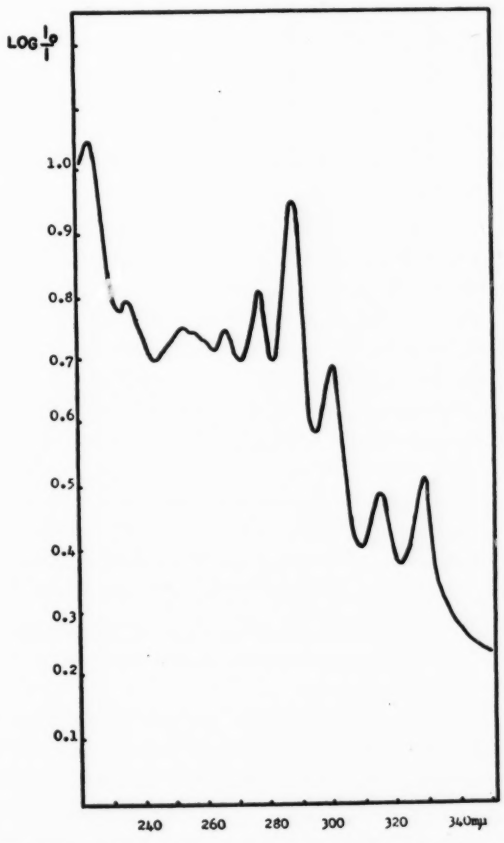


CHART 4.—Absorption spectrum of 1,2-benzpyrene isolated from carbon black. (Compare with Clar [10].)

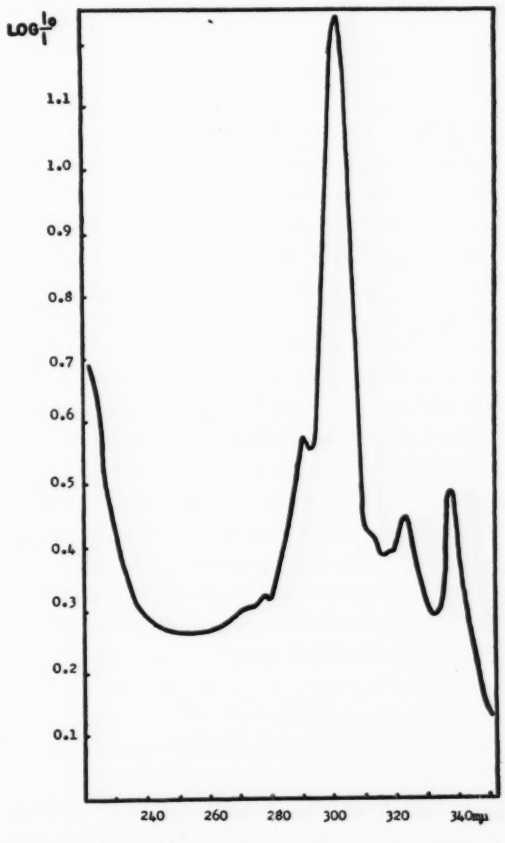


CHART 6.—Absorption spectrum of coronene isolated from carbon black. (Compare with Clar [9].)

covery of hydrocarbons difficult if not impossible, and comparative extraction is difficult because of variation in particle size.

To evaluate the relative importance of the different types of carbon blacks, it should be realized that total production has increased fourfold in the last 15 years, that manufacture of blacks by furnace processes has risen from less than 10 to 50 per cent of the total production, and that 90 per cent of the furnace blacks belong to the "semi-reinforcing" and "high modulus furnace" blacks (26). The blacks of the types from which we have extracted 3,4-benzpyrene have reached a considerable proportion of the total production during recent years.

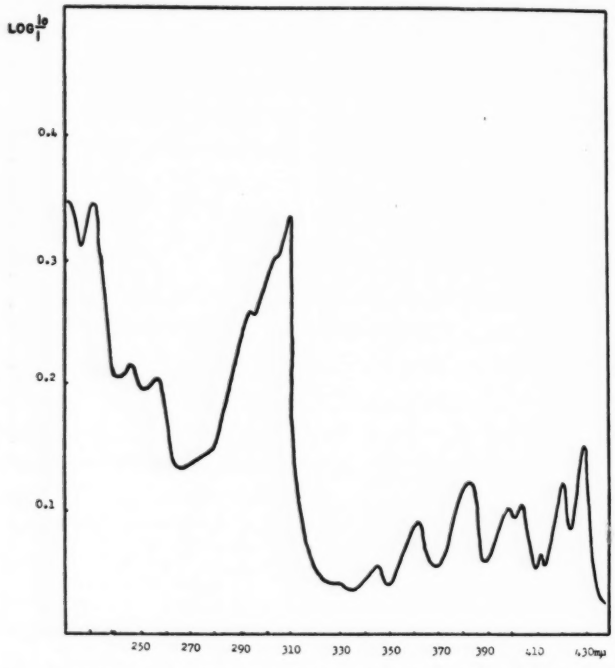


CHART 7.—Absorption spectrum of anthanthrene isolated from carbon black. (Compare with Clar [9].)

Extractable aromatic hydrocarbons were found in carbon blacks of large particle diameter and not in those of small particle size. The particle size of carbon blacks depends on the method and conditions of manufacture. It is possible, because of the adsorption phenomenon to be mentioned, that many, if not all, carbon blacks contain aromatic hydrocarbons and that the differences here reported are due to failure of elution and not due to absence of these substances.

DISCUSSION

Carbon black is practically pure amorphous carbon, the particle size of which ranges in diameter from 10 to 160 mμ. Soot is also amorphous carbon produced by the combustion of a variety of organic substances. Its particle size ranges up to 5,000 mμ. The two terms are used at times to designate identical or closely related products, although soot is the broader and more inclusive term. Almost all persons, and especially those in urban populations, are exposed to soot among other atmospheric dusts. In some localities and in some occupations exposure may be particularly heavy. Soot may also contaminate laboratory equipment and materials in experiments on carcinogenesis, among others. The observation that carbon blacks and soot may contain aromatic polycyclic hydrocarbons, one of which—3,4-benzpyrene—in pure form is a strong carcinogen for animals, while another—1,2-benzpyrene—is a weaker carcinogen (2) would therefore be a matter of wide concern if these substances and compounds should be shown to be harmful in man.

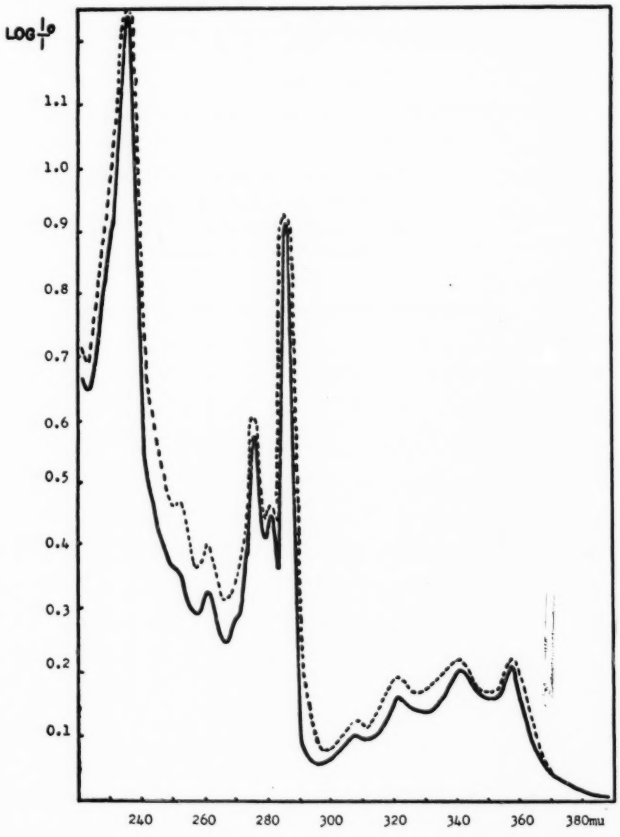


CHART 8: ——— Absorption spectrum of fluoranthene, obtained through the courtesy of R. N. Jones.
------- Absorption spectrum of fluoranthene isolated from carbon black.

Probable origin of these polynuclear aromatic hydrocarbons.—The presence of complex aromatic hydrocarbons, all belonging to one group, in the extracts of carbon blacks, with the exclusion of all other types of hydrocarbons is an interesting phenomenon. Some light can be thrown on the mecha-

nism of formation of these peri-condensed ring systems from information found in the literature. At 1,100°–1,600° C., the temperature reached in production of carbon blacks, all known hydrocarbons tend to decompose into amorphous carbon and hydrogen gas. A study of the products of pyrolysis of the constituents of natural gas as compiled by Ellis (12) gives, in a very much simplified form, the following picture: Methane, the main hydrocarbon constituent of "dry" natural gas, breaks down at 1,100° C. into a methylene radical and hydrogen. This unstable free radical dimerizes to ethylene, which breaks down further to hydrogen gas and to "nascent acetylene," a term coined by Groll (14). The "nascent acetylene" partly decomposes to carbon, hydrogen, and methane and partly polymerizes to simple aromatic hydrocarbons. The aromatic hydrocarbons in part lose hydrogen, forming more complex aromatic hydrocarbons, and in part break down to acetylene, carbon, and hydrogen.

The pyrolysis of ethane, propane, butanes, and pentanes, all constituents of "wet" natural gases used at times in carbon black formation, yields the same type of breakdown products as in the case of methane, but larger quantities of "nascent acetylene" and consequently of aromatic hydrocarbons are formed.

This simplification of the processes involved in the decomposition of hydrocarbons gives a plausible explanation of the side reaction products during carbon black formation. This mechanism may also explain the formation of carcinogenic tars by pyrolysis of all organic materials which were tested by Kennaway (19).

In 1925 Kennaway (20) indicated the type of reaction involved in the formation of strongly carcinogenic tars by pyrolysis of isoprene. He found that polycyclic aromatic hydrocarbons were not produced unless the temperature of pyrolysis reached 750°–820° C. At this temperature he detected benzene, naphthalene, anthracene, phenanthrene, chrysene, and their homologs in the tars which were strongly carcinogenic. The existence of a critical temperature, which must be reached before aromatic hydrocarbons with carcinogenic properties are formed, was mentioned also by Dickens and Weil-Malherbe (11). No evidence of carcinogenicity of extracts of wood soot, produced by them at 400°–450° C., was obtained by skin painting of mice over a period of 2 years.

Identification of carcinogens in soot.—There is ample evidence that at least one—and the most important one, namely, 3,4-benzpyrene—of the aromatic hydrocarbons which we have found in carbon blacks is also present in a number of soots. For example, McDonald and Woodhouse (29) isolated from city dust a fraction in which they identified by its ultraviolet absorption spectrum a compound which they believed to be 3,4-benzpyrene. Hieger (17) exposed pure benzene to city air and observed that it acquired the fluorescence spectrum of 3,4-benzpyrene. By the same physical method 3,4-benzpyrene was detected by Gulden and Tipler (15) in domestic soot in concentrations

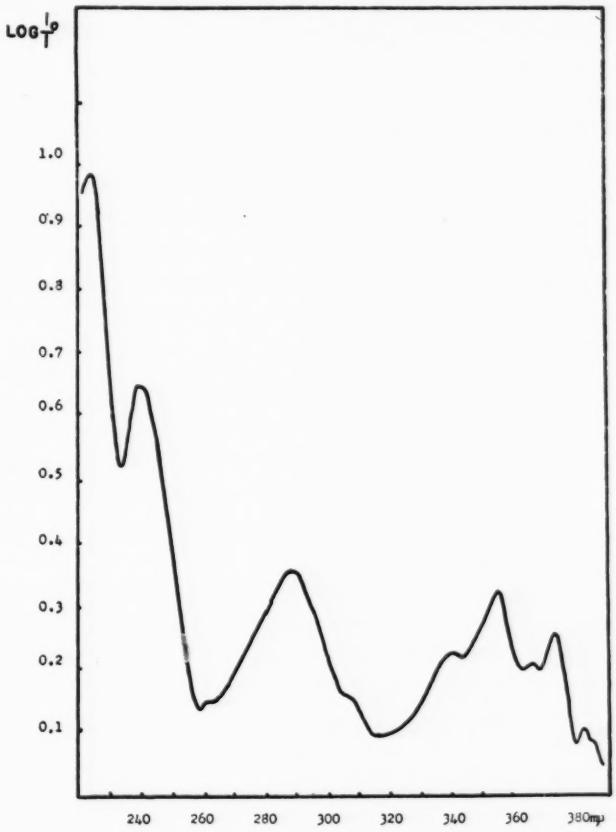


CHART 9.—Absorption spectrum of crystalline compound, which has not as yet been identified, isolated from carbon black.

of 0.03 per cent, and by Waller (44) in the dust of nine British cities in concentrations ranging from 0.02 to 0.5 μg. per cubic meter of air filtered. The quantity of carcinogen in some city air appears therefore to be quite large.

Epidemiological evidence for carcinogenic hazard in soot and atmospheric dusts.—The foregoing evidence of our own on the presence of carcinogenic compounds in carbon blacks together with the evidence summarized from the literature would appear to indicate the possibility of considerable hazard in soot-polluted air. However, a survey of clinical, epidemiological, and statistical evidence in the literature bearing on this problem does not unequivocally indicate a hazard.

If soot and carbon black were to induce human

tumors it would be expected that the resulting tumors would be chiefly located at the points of maximum contact, namely, the skin and respiratory tract, including the lungs. Published statistical studies on the incidence of human cancer in dusty occupations give conflicting results, which may be explained by differences in the composition of the dusts, in the type of contact, and in the method of epidemiological and statistical analysis.

In 1907, Lueke (27) reported a series of cases of epithelioma of the skin in carbon workers. Carbon itself, however, was not definitely established as the cause, because the workers had contact with both ground coke and an oily liquid obtained from tar or pitch. An epidemiological study of cancer mortality and morbidity in a large carbon black manufacturing industry was recently reported by Ingalls (18). He concluded that carbon black workers face no more than the ordinary risks of cancer encountered by other male working populations. It might be pointed out, however, that among the carbon black workers which he studied, only 79 had been employed for 10 years or more, and only 34 for 15 years or longer; furthermore, only 32 were 50 years old or over, and of these only 7 had been employed for 10 years or more: finally, former employees retired for age were not included in the statistics.

A number of statistical reports are available on the incidence of human lung tumors in relation to occupation and environment, but the inhaled atmospheric dusts were only partly soot. The correlation of lung cancer prevalence with occupation seems to incriminate some dusts which are comparatively free of soot, rather than soot itself. For example, Turner and Grace (42) found that steel foundry and furnace workers had the highest incidence of lung cancer, followed by grinders and sand blasters. Brockbank (5), in an analysis of 900 lung tumor cases, observed the predominance of unskilled laborers and dusty occupations. Versluys in Holland (43) found a high incidence of pulmonary cancers in many occupations which appear to have little in common and no undue exposure to soot; he also observed a low incidence in farmers and general laborers, the latter contrary to Brockbank in England. Kennaway and Kennaway in Great Britain (21, 22) remarked on the low incidence of cancer of the respiratory tract in agricultural workers (like Versluys) and in coal miners. Mills and Mills-Porter (30) working with the vital statistics of several American cities reported the mortality from respiratory tract cancer to be greater in areas of high soot fall than in clean districts. Additional comment along this line is provided by the recent discussion by McDonald, Drinker, and Gordon (28). It is apparent that the epidemiological studies do not incriminate soot beyond a doubt as an etiological agent in respiratory tract cancer in man.

Experiments were made to demonstrate carcinogens in extracts of anthracotic human cancerous and cancer-free lungs (40). The weak carcinogenic activity which was demonstrated was nearly as great in extracts of fetal lung as from those of adults. It was concluded, therefore, that no exogenous carcinogen had been demonstrated In recent studies we have attempted to demonstrate the presence of aromatic hydrocarbons in extracts of anthracotic human lungs by spectrophotometry but have failed to find evidence for such compounds.[2]

The evidence for soot as a carcinogen in human cutaneous tumors, however, is stronger than that for lung cancer. Sir Percivall Pott long ago described cancer of the skin of the scrotum and other sites in chimney sweeps and ascribed them to exposure to soot (33, 34). The subsequent confirmation and the development of knowledge on this subject has recently been reviewed by Henry (16). Ingalls (18) has correctly pointed out the possible great difference in carcinogenic hazard between the chimney soots which have a high content of acetone-extractable matter and the industrial carbon blacks with a low tar content.

The available evidence indicates that, while soot is mildly if at all carcinogenic in the respiratory tract of man, it must be considered responsible for some human skin cancer. This difference, as we shall point out later, may be explained by the lack of appropriate solvent for the carcinogen in the respiratory tract and presence of such solvent in the form of sebaceous secretion on the skin.

Experimental evidence for carcinogenic activity of soot.—The experimental evidence on the carcinogenicity of soot and soot-containing dusts in animals is also equivocal. Leitch in 1923 (23) reported unsuccessful attempts to produce cancer of the scrotum with soot, but his experiments continued for only 1 year, and he used the relatively skin-tumor-resistant rats and rabbits. Seelig and Benignus (37) exposed 100 Buffalo strain mice to coal soot substituted for shavings and observed 8 lung tumors as compared to 1 tumor among 50 controls. However, in a later experiment these authors (38), in repeating the experiment with 100 C57 black mice and a 10 per cent mixture of carcinogenic gas works tar in lamp black, induced no lung tumors in the experimental mice, whereas 2 of their controls had tumors. In neither experiment was any

[2] P. E. Steiner, J. Afterman, and H. L. Falk, unpublished data.

skin tumor reported, although the mice lived on the soot. Campbell (7) exposed 75 mice to a moderate cloud of chimney soot 30 times a week during a whole year and observed a lung tumor yield of 20 per cent. As the lung tumor yield in his controls also was as high as 20 per cent in some groups, it is doubtful whether his soot was carcinogenic for lung tissue. In summarizing his experiments on the production of lung tumors in mice, Campbell (8) pointed out the similarity between human and mouse lung cancer as regards the agents which increase the yield of these tumors, the time and age factors, some aspects of susceptibility, and the morphology of the tumors. He placed emphasis on the inorganic constituents rather than on the tars as the carcinogenic agents. McDonald and Woodhouse (29), repeating the work of Seelig and Benignus but using two types of city dusts instead of soot, observed a yield of lung tumors of 22.8 per cent compared to 14.3 per cent in the control group. Schnurer and Haythorn (36) exposed eight rats and one rabbit to a mixture of coal smoke and air during 80 days. The animals developed fibrous reactions around the carbon deposits and lung changes analogous to those of bituminosis as seen in soft-coal miners, but lung tumors were not observed up to 14 months following exposure. Sulman and Sulman (41) studied the carcinogenicity of wood soot from the chimney of a sausage factory. On subcutaneous injection into rats they observed a 16.6 per cent sarcoma yield, while no tumors were induced on intrascrotal implantation of soot or by feeding of the smoked sausage. In conclusion, there has been reported no unequivocal experimental evidence, to our knowledge, for the presence of functioning carcinogens for the respiratory tract in soot.

Experimental evidence for carcinogenic activity in extracts of soot.—Although there is little experimental evidence for carcinogenicity of soots, extracts of soots have consistently shown carcinogenic activity both by skin painting and by subcutaneous injection.

The earliest experiments on this subject were described by Passey (31), who painted an ether extract of household soot from bituminous coal on the skin of mice and observed nine malignant tumors in 18 animals surviving 1 year. In a subsequent paper, Passey and Carter-Braine (32) attempted a fractionation of the soot extract by fractional distillation. The activity was found in the distillate above 190° C. and in the residue. Subcutaneous injection of chimney soot extracts, prepared by Shimkin and Leiter (39), gave a 40 per cent yield of sarcomas in C3H mice. An extract of wood soot was painted on the skin of ten mice in which three neoplasms were observed by Sulman and Sulman after 2 years (41). Extracts of city atmospheric dusts were investigated by Leiter *et al.* for their carcinogenicity on subcutaneous injection into mice at a 50-mg. dose level. Their tars produced 18 sarcomas in 291 mice (24) and 30 sarcomas in 372 mice (25). The activity of these dust extracts showed considerable variation, depending on the locality of collection. A study was made by McDonald and Woodhouse (29) on the lung tumor incidence after skin painting twice a week for 1 year with a purified extract of city dust. Their 50 mice showed a 25 per cent tumor yield, compared to 15.8 per cent in the control group. Campbell (7) painted 24 mice twice a week for 26 weeks with an extract of chimney soot (of the same type used for his inhalation experiments described above) and found 9 papillomas, 7 of which became malignant. In attempting to explain his positive results with the extracts and the negative results with whole soot, he suggested that the presence of the soot decreased the action of the carcinogen.

In contrast to the low activity of whole soot, these tests with extracts demonstrated clear-cut carcinogenic activity. The extracts were active regardless of type of soots or their content of extractable tarry matter which varied from 0.5 to 11.8 per cent of the original soots. This apparent discrepancy between the carcinogenicity of soot and of its extracts needs to be explained.

Lipid solvents and adsorption.—The equivocal evidence for carcinogenic activity in soot by inhalation and dusting experiments in animals and by epidemiological studies on respiratory tract cancer in man, despite demonstrated presence of carcinogens in many soots and of carcinogenic activity in extracts of soots, requires an explanation. Two immediately come to mind. They are (*a*) that an adequate solvent for extraction of the carcinogen from the carbon may be lacking at some sites of contact and (*b*) that the carcinogen may be held by adsorption so firmly as to be biologically inactive.

Two points of evidence for the importance of the lipid solvent factor have already been mentioned. First, soots are carcinogenic on the skin of man where sebaceous secretions are present and questionably active in the respiratory tract where such secretions are absent. Second, lipid solvent extracts of soot have consistently produced tumors when tested in animals. It is possible that in the human respiratory tract the carcinogen was present but did not make proper contact with the cells for lack of solvent.

There is also evidence for the adsorption hypothesis. If a carcinogenic hydrocarbon is added to

some carbon blacks under special conditions, it can be recovered incompletely or not at all. This phenomenon is the subject of the paper which follows. Furthermore, this adsorption phenomenon was easily demonstrated in the test tube by another method. When a furnace black from which hydrocarbons could be eluted was mixed with a channel black from which none could be extracted, in a proportion of 3:1, aromatic hydrocarbons were not extractable from the mixture. This phenomenon is not clarified by the experiments of Andervont and Lorenz (1). When they injected a mixture of charcoal and 1,2,5,6-dibenzanthracene, they observed the same yield of sarcomas at the site of subcutaneous injection in the experimental as in the 1,2,5,6-dibenzanthracene controls, but fewer lung tumors in the former. They attributed the latter difference to adsorption of the chemical on the charcoal. Examination of their methods, however, shows that they used a ratio of charcoal to carcinogen of 2:1. This is a much larger amount of carcinogen than could have been adsorbed to the charcoal, so that their explanation is not fully adequate.

There is much chemical but little biological evidence for the importance of the adsorption phenomenon. Extensive biological tests bearing on these points are now un ler way.

SUMMARY

1. Benzene extracts of some carbon blacks were found to contain 3,4-benzpyrene, a strong carcinogen for animals; 1,2-benzpyrene, a weak carcinogen; and five other related aromatic hydrocarbons: pyrene, fluoranthene, 1,12-benzperylene, anthanthrene, and coronene.

2. These compounds possess peri-condensed ring systems in which carbon atoms are shared by three rings. They contain the smallest possible proportion of hydrogen to carbon for molecules of this type and size. A theory of their formation is presented.

3. Furnace blacks, with an average particle diameter of 80 mμ or more, possessed all 7 of the aromatic hydrocarbons; those with particle size below 50 had few or none. A rough correlation existed between yield of extractable aromatic hydrocarbons and average particle diameter of the carbon blacks.

4. These hydrocarbons were not detected in any channel blacks, which have small particle diameter averaging from 10 to 30 mμ, and are made by a different process.

5. Based on these observations an interpretation of the apparently contradictory reports in the literature on the carcinogenicity of soot is possible. 3,4-Benzpyrene seems to be the principal carcinogenic agent. This hydrocarbon can be extracted from many soots with lipid solvents, and these extracts produce tumors in animals at the site of application. Soot itself appears to be carcinogenic on skin but possibly not on respiratory tract epithelium. It is suggested that this difference in behavior is explained by the presence of lipid solvent (the sebaceous secretions) in the skin and its absence in the respiratory tract.

6. It is further suggested that the differences in extractable aromatic hydrocarbons from carbon blacks can be explained by the phenomenon of adsorption.

REFERENCES

1. Andervont, H. B., and Lorenz, E. Dibenzanthracene Tumors in Mice: The Production of Subcutaneous and Pulmonary Tumors by 1:2:5:6-Dibenzanthracene Adsorbed on Charcoal. U.S. Pub. Health Rep., **52**:1931–40, 1937.
2. Barry, G.; Cook, J. W.; Haslewood, G. A. D.; Hewett, C. L.; Hieger, I.; and Kennaway, E. L. The Production of Cancer by Pure Hydrocarbons. Part III. Proc. Roy. Soc., London, s.B, **117**:318–51, 1935.
3. Berenblum, I. 3-4-Benzpyrene from Coal Tar. Nature, **156**:601, 1945.
4. Brass, K., and Clar, E. Anomale Halogenide des Anthanthrens und Versuche zur Darstellung von Monohalogeniden des Peri-naphthindenons. V. Mitteil. Über anomale Halogenide. Ber. deutsch. chem. Gesellsch., **72**:1882–84, 1939.
5. Brockbank, W. Occupational Incidence of Primary Lung Cancer. Quart. J. Med., **25**:31–40, 1932.
6. Campbell, A. W.; Cromwell, N. H.; and Hager, J. J. Isolation and Identification of Acenaphthylene and Pyrene from the Products of the Pyrolysis of Natural Gas. J. Am. Chem. Soc., **58**:1051–52, 1936.
7. Campbell, J. A. Carcinogenic Agents Present in the Atmosphere and Incidence of Primary Lung Tumors in Mice. Brit. J. Exper. Path., **20**:122–32, 1939.
8. ———. Lung Tumours in Mice and Man. Brit. M. J., **1**:179–82, 1943.
9. Clar, E. Aromatische Kohlenwasserstoffe, polycyclische Systeme. Berlin: Springer-Verlag, 1941.
10. ———. Neue Synthesen von Benzologen des Pyrens und Benzanthrens. Aromatische Kohlenwasserstoffe. XLI. Mitteil. Ber. deutsch. chem. Gesellsch., **76**:609–21, 1943.
11. Dickens, F., and Weil-Malherbe, H. Investigation into the Possible Carcinogenic Activity of Wood Smoke. Cancer Research, **2**:680–84, 1942.
12. Ellis, C. The Chemistry of Petroleum Derivatives, Volume II. New York: Reinhold Publishing Corp., 1937.
13. Falk, H. L.; Steiner, P. E.; Goldfein, S.; Breslow, A.; and Hykes, R. Carcinogenic Hydrocarbons and Related Compounds in Processed Rubber. Cancer Research, **11**:318–24, 1951.
14. Groll, H. P. A. Vapor-Phase Cracking. Indust. & Eng. Chem., **25**:784–98, 1933.
15. Gulden, F., and Tipler, M. M. Experiments on the Identification of 3,4-Benzpyrene in Domestic Soot by Means of the Fluorescent Spectrum. Brit. J. Cancer, **3**:157–60, 1949.
16. Henry, S. A. Cancer of the Scrotum in Relation to Occupation. London: Oxford University Press, 1946.
17. Hieger, I. Carcinogenic Substances in Human Tissues. Cancer Research, **6**:657–67, 1946.

18. INGALLS, T. N. Incidence of Cancer in the Carbon Black Industry. Arch. Ind. Hyg. & Occup. Med., **1**:662–76, 1950.
19. KENNAWAY, E. L. Experiments on Cancer-producing Substances. Brit. M. J., **2**:1–4, 1925.
20. ———. The Formation of Cancer-producing Substances from Isoprene (2-Methyl-Butadiene). J. Path. & Bact., **27**:233–38, 1924.
21. KENNAWAY, E. L., and KENNAWAY, N. M. A Further Study of the Incidence of Cancer of the Lung and Larynx. Brit. J. Cancer, **1**:260–98, 1947.
22. KENNAWAY, N. M., and KENNAWAY, E. L. A Study of the Incidence of Cancer of the Lung and Larynx. J. Hyg., **36**: 236–67, 1936.
23. LEITCH, A. The Experimental Inquiry into the Causes of Cancer. Brit. M. J., **2**:1–7, 1923.
24. LEITER, J.; SHIMKIN, M. B.; and SHEAR, M. J. Production of Subcutaneous Sarcomas in Mice with Tars Extracted from Atmospheric Dusts. J. Nat. Cancer Inst., **3**:155–65, 1943.
25. LEITER, J., and SHEAR, M. J. Production of Tumors in Mice with Tars from City Air Dusts. J. Nat. Cancer Inst., **3**:167–74, 1943.
26. LOTT, F. S.; COLBY, D. S.; and BACKUS, H. Carbon Black. Minerals Yearbook 1948, U.S. Department of the Interior, Bureau of Mines, pp. 200–11. Washington: U.S. Government Printing Office, 1950.
27. LUEKE, A. W. Epithelioma in Carbon Workers. Cleveland M. J., **6**:199–202, 1907.
28. MCDONALD, J. C.; DRINKER, P.; and GORDON, J. E. The Epidemiology and Social Significance of Atmospheric Smoke Pollution. Am. J. Med. Sc., **221**:325–42, 1951.
29. MCDONALD, S., JR., and WOODHOUSE, D. L. On the Nature of Mouse Lung Adenomata with Special Reference to the Effects of Atmospheric Dusts on the Incidence of These Tumours. J. Path. & Bact., **54**:1–12, 1942.
30. MILLS, C. A., and MILLS-PORTER, M. Health Costs of Urban Air Pollution. Occup. Med., **5**:614–33, 1948.
31. PASSEY, R. D. Experimental Soot Cancer. Brit. M. J., **2**: 1112–13, 1922.
32. PASSEY, R. D., and CARTER-BRAINE, J. Experimental Soot Cancer. J. Path. & Bact., **28**:133–44, 1925.
33. POTT, P. Chirurgical Observations. London, 1775.
34. ———. The Chirurgical Works. Cancer Scroti, **3**:177–83. London: Wood & Innes, 1808.
35. REHNER, J., JR. The Isolation and Identification of Fluoranthene from Carbon Black. J. Am. Chem. Soc., **62**: 2243–44, 1940.
36. SCHNURER, L., and HAYTHORN, S. R. The Effects of Coal Smoke of Known Composition on the Lungs of Animals. Am. J. Path., **13**:799–809, 1937.
37. SEELIG, M. G., and BENIGNUS, E. L. Coal Smoke Soot and Tumors of the Lung in Mice. Am. J. Cancer, **28**:96–111, 1936.
38. ———. Lung Tumor Development in a Resistant Strain of Mice Subjected to Inhalation of Soot. *Ibid.*, **34**:391–98, 1938.
39. SHIMKIN, M. B., and LEITER, J. Induced Pulmonary Tumors in Mice. III. The Role of Chronic Irritation in the Production of Pulmonary Tumors in Strain A Mice. J. Nat. Cancer Inst., **1**:241–54, 1940.
40. STEINER, P. E.; STANGER, D. W.; and BOLYARD, M. N. A Search for Carcinogenic Substances in Carcinomatous Human Lungs. Arch. Path., **43**:590–601, 1947.
41. SULMAN, E., and SULMAN, F. The Carcinogenicity of Wood Soot from the Chimney of a Smoked Sausage Factory. Cancer Research, **6**:366–67, 1946.
42. TURNER, H. M., and GRACE, H. G. An Investigation into Cancer Mortality among Males in Certain Sheffield Trades. J. Hyg., **38**:90–103, 1938.
43. VERSLUYS, J. J. Cancer and Occupation in the Netherlands. Brit. J. Cancer, **3**:161–85, 1949.
44. WALLER, R. E. Atmospheric Pollution in Relation to Cancer of the Lung. Benzpyrene. British Empire Cancer Campaign, 27th Annual Report, pp. 98–99, 1949.

The Adsorption of 3,4-Benzpyrene and Pyrene by Carbon Blacks*

HANS L. FALK and PAUL E. STEINER

(*Department of Pathology, University of Chicago, Chicago 37, Ill.*)

In the previous paper it was reported that up to seven different aromatic polycyclic hydrocarbons, including 3,4-benzpyrene, were extracted with benzene from most carbon blacks made by the furnace process, but from no channel blacks (3). This difference cannot be explained on the basis of differences in raw materials. Soot formed in the laboratory from gas, which is similar to that used in the channel process, also yielded aromatic hydrocarbons on extraction. Both the channel and furnace processes use a temperature so high (1,100°–1,600° C.) that nearly complete decomposition of the raw materials into amorphous carbon and hydrogen is assured. However, a few radicals of the nature of "nascent acetylene" might form and polymerize to produce the observed aromatic hydrocarbons. These compounds would, therefore, be expected to be formed in both processes unless special precautions are taken which have not been publicized. Failure to find the aromatic hydrocarbons in the channel blacks, therefore, constituted something of a mystery.

One of the most important differences between the hydrocarbon-free channel blacks and the furnace blacks rich in extractable hydrocarbons seems to be the smaller average particle size of the former. The furnace blacks are said to vary from 30 to 160 mμ in average particle diameter; the channel blacks from 10 to 30 mμ. It is well known that the smaller the average particle size, the larger is the surface area, and that surface area is one of the factors responsible for the adsorptive power of active carbons. Hassler (4) described the physical and chemical behavior of active carbon in detail. A study of the adsorption of aromatic hydrocarbons having peri-condensed ring systems on carbon blacks of varying particle size was therefore undertaken as a possible explanation for the differences in polycyclic aromatic hydrocarbon content of channel and furnace blacks.

* This investigation was aided by a research grant from the Committee on Growth acting for the American Cancer Society, and by an institutional grant from the American Cancer Society.

Received for publication September 17, 1951.

Another problem is presented by the discrepancy between the ease with which 3,4-benzpyrene has been demonstrated in soots (in large quantities in some instances), and the apparent low carcinogenic activity of soots in man and experimental animals. This problem has also been discussed in the preceding paper (3), in which it was pointed out that low or absent carcinogenicity of soots might be explained either by the absence in the body of a solvent adequate for this class of carcinogens, by adsorption of the carcinogen to soot, or by both. Some evidence was cited for both hypotheses. The seemingly greater carcinogenic activity of soot on the human sebaceous-rich skin than on the respiratory tract epithelium speaks for the former; the adsorption phenomenon was simply shown by mixing benzpyrene-containing and benzpyrene-free carbon blacks after which process no hydrocarbon was recoverable from the mixture. It is conceivable that a similar mixing of carbon blacks might occur in air or lungs. The present paper provides additional information on this adsorption phenomenon.

EXPERIMENTAL RESULTS

The adsorption of aromatic hydrocarbons from solution by carbon black.—One hundred mg. each of ten different samples of carbon black, averaging from 10 to 80 mμ in particle diameter, were placed in 15-ml. graduated centrifuge tubes, each containing 5 ml. of redistilled benzene. One-ml. portions of benzene containing 5 μg. of 3,4-benzpyrene were added to each, and the mixtures were agitated with stirring rods for about 15 seconds. After centrifugation the supernatant fluid was transferred to a Beckman cup, and the ultraviolet absorption spectrum was read between the wavelengths of 355 and 400 mμ. The concentration of 3,4-benzpyrene was calculated by using the absorption maximum at 387 mμ. The solutions were returned to the centrifuge tubes, an additional ml. of the benzpyrene solution was added to each tube, the volume of the solutions was reduced to the previous level by evaporation under a stream of nitrogen, and the mixing, centrifugation, and

spectrophotometry were again carried out. This process was repeated (later on with larger increments of 3,4-benzpyrene) until the absorption spectrum of the added hydrocarbon could be clearly detected.

The results are summarized in Table 1. When 3,4-benzpyrene was first added to some carbon blacks it promptly disappeared from the solution because of adsorption. In others only a portion was detectable. In still others more benzpyrene was detected than had been added. This behavior was correlated with average particle diameter of the blacks; the smaller average-sized blacks adsorbed more than the larger. As more and more benzpyrene was added, a point was eventually reached at which it was no longer completely adsorbed. The point at which this occurred varied with the particle size of the blacks. With the smallest-sized carbon black (10 mμ), 200 μg. of 3,4-benzpyrene was completely adsorbed on 100 mg. of the black; when 500 μg. was added, only 1 μg. was detected in solution. In the largest-sized carbon black (80 mμ), not only was adsorption not observed, but actual elution of part of its own content of 3,4-benzpyrene occurred. No attempt had been made to alter the hydrocarbon content of these ten carbon blacks prior to their use in this experiment. As was shown in the preceding paper, the content of extractable aromatic hydrocarbons varies with particle size (3). The present experiments, therefore, tested only the remaining adsorptive power of the blacks.

TABLE 1

ADSORPTION OF 3,4-BENZPYRENE BY DIFFERENT CARBON BLACKS

Code no. of sample	Av. particle diameter (mμ)	Amount of 3,4-benzpyrene added* (μg.) Quantity of 3,4-benzpyrene detected (μg.)												
		5	10	15	20	25	50	100	150	200	250	300	400	500
1	10	0	0	0	0	0	0	0	0	0	0.2	0.4	0.7	1
2	14	0	0	0	0	0	0	0	0	0	1	2	4	7
3	17	0	0	0	0	0	0	0	1.5	3				
4	25	0.5	1	1.7	2	2.6	6							
5	29	0.7	1	2	2.5	3	8							
6	30	0.6	1	1.5	2	2.4	7							
7	39			3	4	5	14							
8	50	6	9											
9	80	20	26											
10	80	19	25											

* The 3,4-benzpyrene was tested, in every instance, in 100 mg. of the carbon black in 6 ml. of benzene.

The elution of aromatic hydrocarbons from carbon black.—The question arose whether these aromatic hydrocarbons could be recovered by elution from the carbon blacks once they were adsorbed. Samples (100 mg.) of a channel black with an average particle diameter of 14 mμ were placed into each of four 15-ml. centrifuge tubes. Nine ml. of redistilled benzene was added, and benzene solutions were then introduced containing 0.4 mg., 0.8 mg., 1.2 mg., and 2.4 mg. of 3,4-benzpyrene, respectively, in 1 ml. The suspensions were stirred, centrifuged, and the supernatants were removed and analyzed spectrophotometrically to determine the quantity of 3,4-benzpyrene in solution. Fresh 10-ml. aliquots of benzene were then added to the carbon blacks in the centrifuge tubes, and the process was repeated. After ten extractions, the pattern of elution was well established, and variations were then introduced to study the effects of temperature, time of stirring, and the volume of the solvent on extraction.

The results are summarized in Table 2 and Chart 1. The amount of extractable benzpyrene decreased slowly from one extraction to the next. When large quantities of benzpyrene had been adsorbed on the carbon black, a greater percentage could be eluted. By the end of the tenth extraction, the percentage recoveries were 7.1, 9.8, 13.6, and 21.8, respectively, in the four experimental samples. The quantity of the eluate was directly proportional to the volume of eluant, and to the temperature between 40° and 90° C. The period of agitation, however, had no effect on the quantity of hydrocarbon eluted in a range from 15 to 300 seconds.

Two solvents, ether and methanol, were found to be inferior to benzene as eluants. They eluted traces of benzpyrene only after the concentration of hydrocarbon added to carbon black had reached 0.8 per cent. Three times as much hydrocarbon could be held by adsorption on carbon black against these two solvents as compared with benzene; ether liberated only one-fifteenth of the quantity eluted by benzene, and methanol was still less efficient.

Experiments with pyrene.—The experiments were repeated by substituting pyrene for 3,4-benzpyrene. The adsorption of pyrene on the same

ten carbon blacks was found to be similar to that of 3,4-benzpyrene. The elution pattern of pyrene from carbon black by benzene, ether, and methanol, and the effects of temperature and volume on elution paralleled those for 3,4-benzpyrene.

DISCUSSION

From these data the observed difference in hydrocarbon content of extracts of channel and furnace blacks could be explained as caused by the phenomenon of adsorption. Even the best eluant here studied, under most favorable conditions, did not liberate benzpyrene from a channel black averaging 10 mμ in particle diameter, either before or after 0.5 mg. of benzpyrene had been added to 100 mg. of the black. Such a black appeared devoid of carcinogenic hydrocarbon. Yet 0.5 per cent had been added. It is reasonable to suspect that an additional unknown quantity may previously have been present in this channel black because of the conditions of manufacture (3). The differences in amount of hydrocarbon extractable from various carbon blacks reported previously (3) may only be due to a difference in the adsorptive capacity of the blacks. A carbon black of 10 mμ average particle diameter from which no carcinogen is extractable might conceivably contain as much as the black of 80 mμ from which much is obtained.

TABLE 2

ELUTION OF 3,4-BENZPYRENE BY BENZENE FROM A CHANNEL BLACK

No. extractions	Conditions of elution: Volume of benzene (ml.)	Temperature (°C.)	Time (min.)	Mg. of 3,4-benzpyrene adsorbed on 100 mg. channel black: 0.4	0.8	1.2	2.4
				Cumulative percentage of 3,4-benzpyrene recovered on successive extractions with benzene			
1	10	20	1	1.1	1.0	1.7	2.3
2	10	20	1	2.2	2.1	3.2	4.9
3	10	20	1	3.2	3.2	4.7	7.5
4	10	20	1	4.1	4.3	6.1	9.8
5	10	20	1	4.8	5.3	7.5	12.0
6	10	20	1	5.3	6.2	8.7	14.2
7	10	20	1	5.8	7.1	10.0	16.2
8	10	20	1	6.3	8.0	11.2	18.1
9	10	20	1	6.7	8.9	12.4	20.0
10	10	20	1	7.1	9.8	13.6	21.8
11	5	20	1	7.3	10.2	14.2	22.6
12	5	40	1	7.6	10.7	14.9	23.4
13	5	70	1	8.0	11.4	15.9	24.7
14	5	90	1	8.5	12.3	17.3	26.5
15	5	20	5	8.7	12.7	17.9	27.3
16	5	70	5	9.1	13.4	18.9	28.6

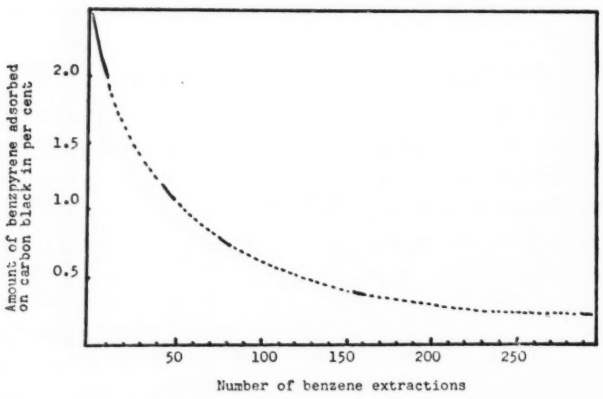


CHART 1.—A pattern of elution of 3,4-benzpyrene from a channel black. The four solid portions each represent ten actual consecutive extractions, as shown in Table 2. The dotted line is an extrapolation of the rate of decrease of the elutable portion.

The adsorption phenomenon makes quantitative determinations of amounts of carcinogen present in carbon black very difficult. One extraction does not indicate the amount present. Even the tenth extraction of a furnace black yielded nearly as much aromatic hydrocarbon as the first. After a known amount of benzpyrene was added to a channel black, ten consecutive extractions recovered only a small proportion of the total added.

The adsorption phenomenon might have many points of significance. It might explain the apparent low carcinogenicity of soots in man. In the respiratory tract an efficient solvent to elute the carcinogen from the particles of soot is probably not present. Even when a lipid solvent is available, as in the skin, the presence of small-sized particles of soot could prevent the extraction of carcinogens from the larger-sized soot particles by adsorption. Adsorption might also explain discrepancies observed in experimental carcinogenesis based on method of administration. Negative results were frequently observed with whole soot as the carcinogenic agent, and positive results were uniformly obtained with extracts of soot.

This phenomenon might provide a method for the disposal of carcinogen-containing waste prod-

ucts, especially if it is eventually shown that adsorption also confers biological inactivity to the carcinogen-adsorbant complex. It might also indicate desirable changes in the method of manufacture or utilization of carbon blacks (1, 2).

SUMMARY

1. The adsorption of 3,4-benzpyrene and pyrene on ten different carbon blacks with an average particle diameter varying from 10 to 80 mμ was found to be inversely proportional to particle size.

2. The elution of these hydrocarbons from the carbon blacks depended on the nature of the black, the nature and volume of the solvent, and the temperature. With each extraction only a fraction of the total adsorbate could be eluted.

3. It is suggested that the observed difference in quantity of aromatic hydrocarbons extracted from channel and furnace blacks is due to greater adsorption on the former than on the latter. The adsorption phenomenon may also play a significant role in the understanding of the low carcinogenicity of soot in man. Other possible applications are also discussed.

REFERENCES

1. FALK, H. L.; STEINER, P. E.; and GOLDFEIN, S. Carcinogenic Hydrocarbons in Processed Rubber and in Carbon Black. Cancer Research, **11**:247, 1951.
2. FALK, H. L.; STEINER, P. E.; GOLDFEIN, S.; BRESLOW, A.; and HYKES, R. Carcinogenic Hydrocarbons and Related Compounds in Processed Rubber. Cancer Research, **11**: 318–24, 1951.
3. FALK, H. L., and STEINER, P. E. The Identification of Aromatic Polycyclic Hydrocarbons in Carbon Blacks. Cancer Research, **12**:30–39, 1952.
4. HASSLER, J. W. Active Carbon. The Modern Purifier, pp. 61–86. New York: Githens-Sohl Corporation, 1941.

Metabolism of Neoplastic Tissue
II. A Survey of Enzymes of the Citric Acid Cycle in Transplanted Tumors*

CHARLES E. WENNER,† MORRIS A. SPIRTES, AND SIDNEY WEINHOUSE

(The Lankenau Hospital Research Institute and the Institute for Cancer Research, and the Department of Chemistry, Temple University, Philadelphia, Pa.)

Recent isotopic tracer studies of tumor metabolism indicated that the neoplastic cell can carry out the complete oxidation of glucose and fatty acids via the citric acid cycle (34, 35). In view of a strong body of evidence against the occurrence of the Krebs cycle in tumors (18, 19), further information bearing on this problem seemed desirable. Accordingly, a survey was undertaken of various transplanted neoplasms for their content of citric acid cycle enzymes. In order to obtain a "profile" of enzymatic activity, approximate assays were carried out of those enzymes for which straightforward methods were available; these included malic, isocitric, and α-ketoglutaric dehydrogenases, the "condensing" enzyme, and the hydrases, fumarase and aconitase. The peripheral enzymes, lactic dehydrogenase and oxalacetic carboxylase, were also assayed, but succinic dehydrogenase, having been thoroughly investigated and found to be present in tumors by others (24, 25), was omitted from the present investigation. All the tumors were found to possess the enzymatic equipment for the citric acid cycle.

METHODS AND RESULTS

Tumors.—The neoplasms employed in this study were described in previous papers (33, 35). They were implanted subcutaneously and used in the experiments when they attained a diameter of approximately 1 cm. Assays were carried out either on homogenates of the whole tissue or on extracts of acetone powders. All assays were carried out in the zero order range of substrate concentration and with proportionality of enzyme activity and tissue concentration—these conditions having been established by preliminary experiments. Details of the procedures are given in the individual sections and in the table headings. The preparations of homogenates will be described in the individual assays. The acetone powder extracts were prepared as follows: the freshly dissected tissue (pooled from four to six animals) was homogenized for 1 minute at 2° C. in a Waring Blendor with 2 volumes of acetone. The homogenate was filtered with suction, and the residue was resuspended in 10 volumes of acetone and refiltered with suction. The tightly packed cake was powdered in a mortar, air-dried on filter paper, then dried thoroughly in a vacuum desiccator over sulfuric acid and paraffin, and stored at less than 5°. The extract was prepared by stirring 1 gm. of the acetone powder with 10 ml. of water for 10 minutes at 40° C. and by centrifuging at 3,000 r.p.m. for 20 minutes in the angle head of a No. 2 refrigerated International Centrifuge. The opalescent solution was used as such or was further diluted if necessary.

Substrates and other materials.—Pyruvic, malic, citric, and α-ketoglutaric acids, and adenosine triphosphate were commercial products. Lithium acetyl phosphate, 60 per cent pure, was obtained through the kindness of Drs. Stern and Ochoa. Oxalacetic acid, 90 per cent pure by aniline citrate assay (5), was prepared from the ester by the procedure of Krampitz and Werkman (9). Isocitric acid was prepared by catalytic reduction of triethyl oxalosuccinate, synthesized according to the method of Wislicenus and Waldmüller (38). The reduction was carried out with platinum at room temperature at 3–4 atmospheres of hydrogen, and the reduced product was saponified with the calculated quantity of sodium hydroxide. The final

* This work was aided by grants from the American Cancer Society, on recommendation by the Committee on Growth of the National Research Council; the National Cancer Institute of the National Institutes of Health, Public Health Service; and the U.S. Atomic Energy Commission, Contract No. AT(30-1)777.

A preliminary report of a portion of this work has appeared (36).

† This study will constitute part of a thesis to be presented by C. E. Wenner to the Graduate School of Temple University in partial fulfillment of the requirements for the Ph.D. degree.

Received for publication September 24.

product consisted of a mixture of four isomers, D- and L-isocitric and D- and L-alloisocitric acids. As expected, only ¼ of the product was acted upon by aconitase, the active isomer being presumably D-isocitric.

Reduced diphosphopyridine nucleotide was prepared according to the method of Ohlmeyer (17) from commercial cozymase assaying 33 per cent pure by the enzymatic method of Racker (22) employing alcohol dehydrogenase. Triphosphopyridine nucleotide, assaying 40 per cent pure by the Zwischenferment assay (32), was prepared by the method of LePage and Mueller (11).

Condensing enzyme.—Of all the reactions of the cycle, the one least understood until quite recently is the condensation leading to formation of the 6-carbon acid. Recent studies have clarified this process, however (16, 26, 29), and the reaction can now be formulated, according to Stern and Ochoa (28), as follows:

$$\text{Acetyl-Coenzyme A} + \text{oxalacetate} \longrightarrow \text{citrate} + \text{Coenzyme A.} \quad (1)$$

An impure concentrate of acetyl-CoA has been obtained by Lynen and Reichart (12); and Ochoa and co-workers[1] have found that this substance can condense with oxalacetate to yield citrate in the presence of the crystalline condensing enzyme. For assay purposes the acetyl-CoA may be generated *in situ*, either by an acetate-activating factor which, according to the original procedure of Stern and Ochoa (27), catalyzes the formation of acetyl-CoA from acetate, CoA and ATP; or, more conveniently, by means of the following reaction catalyzed by a transacetylating enzyme present in *E. Coli* (26):

$$\text{Acetyl phosphate} + \text{CoA} \xrightarrow{E.\ Coli} \text{Acetyl-CoA} + \text{phosphate.} \quad (2)$$

The latter procedure was used in the present study. According to equation (3), representing a summation of equations (1) and (2), citric acid,

$$\text{Acetyl phosphate} + \text{CoA} + E.\ Coli\ \text{extract (}condensing\ enzyme\text{)} + \text{oxalacetate} \longrightarrow \text{citrate} + \text{CoA} + \text{phosphate}, \quad (3)$$

is formed in the presence of the condensing enzyme and *E. Coli* extract from a mixture of Acetyl phosphate, coenzyme A, and oxalacetate. The *E. Coli* extract was prepared, according to directions of Drs. Stern and Ochoa,[2] from lyophilized cells supplied by these investigators. Cells (0.5 gm.) were intimately mixed with 0.5 gm. of alumina (Alcoa No. A301, 325 mesh) by grinding together in a mortar for a few minutes at room temperature. The mixture was transferred to a Lusteroid centrifuge tube of 10-ml. capacity and stirred with 0.5 ml. of M/50 phosphate buffer, pH 7. The suspension was centrifuged for 20 minutes in a Sorvall centrifuge at 12,000 r.p.m. The supernatant fluid, which contained the transacetylase, was kept refrigerated at all times and was freshly prepared each day of use. Sufficient coenzyme A is present in this extract so that no further additions are required.

Since only small quantities of some of the materials were available, only a few assays were performed. However, the data, given in Table 1, convincingly demonstrate the presence of the condensing enzyme in three mouse tumors in amounts at least as high as in mouse liver. The activities of these neoplastic tissues were of the same order as pigeon liver, reported by Stern and Ochoa (26), and were of a magnitude similar to that obtained by these investigators for other normal tissues (29).

TABLE 1

CONDENSING ENZYME ASSAY

The components of the assay system were: 1M phosphate buffer, pH 7, 0.025 ml.; 0.08 M $MgCl_2$, 0.05 ml.; 0.2 M cysteine (neutralized), 0.05 ml.; 0.14 M oxalacetic acid, 0.05 ml.; 0.1 M synthetic acetyl phosphate, 0.1 ml.; *E. Coli* extract, 0.20 ml.; acetone powder extract of the tissue and water to make a total volume of 1.00 ml. After incubation in small test tubes for 10 minutes at 25° the reaction was stopped by adding trichloroacetic acid to a final concentration of 5 per cent. An aliquot of the supernatant solution was removed for citric acid determination according to Natelson *et al.* (15).

Tissue	μM citrate produced 10 min. × 100 mg. acetone powder
Normal mouse liver	1.53
Mouse mammary carcinoma	3.30
Mouse hepatoma	2.90
Mouse rhabdomyosarcoma	1.45

Aconitase and fumarase.—For assay of the hydrases, advantage was taken of the recent spectrophotometric method described by Racker (21). With citrate or malate as the substrate, aconitase or fumarase activity is measured by the appearance of absorption at 240 mμ of the corresponding unsaturated acids, *cis*-aconitic or fumaric. The test system contained, in a total volume of 3 ml., 0.05 M phosphate buffer, pH 7.4, an aliquot of the tissue, and the substrate. For aconitase assay the enzyme was prepared by homogenizing the fresh tissue with 20 volumes of 0.1 M phosphate buffer, pH 7.4, and centrifuging off the residue at 3,000 r.p.m. Fumarase was assayed in an extract of an acetone powder prepared as described in the condensing enzyme assay. The reactions were carried

[1] Private communication.

[2] We are greatly indebted to these investigators for generous gifts of acetyl phosphate, lyophilized *E. Coli*, and details of the condensing enzyme assay prior to publication.

out in quartz absorption cells, with the use of the Beckman DU spectrophotometer. A unit of enzyme activity is defined, according to Racker (21), as the amount causing an increase in optical density (log $[I_0/I]$) of 0.001 per minute at room temperature, 22°–26°. The values obtained were corrected for changes in a control cell with substrate omitted.

As shown in Table 2, both aconitase and fumarase are present in neoplastic tissues. Though the fumarase content in tumors was comparable to that in normal tissues, aconitase activity was quite low. Since aconitase is a very unstable enzyme (3), it is not certain whether the low values observed represent a real deficiency or whether its inactivation was more rapid in the tumor than in normal tissue.

TABLE 2

ACONITASE AND FUMARASE IN NORMAL AND NEOPLASTIC TISSUE

The test systems consisted of the following: aconitase, 0.05 M phosphate buffer, pH 7.4; sodium citrate, 0.03 M; tissue extract and H_2O to a total volume of 3 cc.; fumarase, 0.05 M phosphate buffer, pH 7.4; sodium malate, 0.003 M; tissue extract and H_2O to a total volume of 3 cc.

Tissue	Aconitase Units/mg dry wt of tissue	Fumarase Units/mg dry wt of acetone powder
Normal:		
Liver (mouse)	33	132
Heart (rat)	72	96
Liver (rat)	25	66
Kidney (rat)	50	62
Muscle (rat)	12	
Neoplastic:		
Hepatoma (rat)		133
Hepatoma (mouse)	8.3	62
Rhabdomyosarcoma (mouse)	2.2–5.0*	50
Mammary adenocarcinoma (mouse)	3.1–5.9*	50

* Range of three determinations.

Dehydrogenases.—In view of the long-held belief (31) that tumors are deficient in their capacity for the oxidation of carbohydrate, it was of special interest to test the neoplasms for their content of the dehydrogenases of the citric acid cycle, malic and isocitric dehydrogenases. Lactic dehydrogenase was included in this study because of its possible role in the high aerobic glycolysis of tumors. The method employed in this study was that of Mehler *et al.* (13), which provides a direct assay of the dehydrogenase by measuring the rate of oxidation or reduction of the specific coenzyme. The reactions measured are given in the equations below:

$$\text{Pyruvate} + \text{DPN-H}_2 \rightarrow \text{Lactate} + \text{DPN} \quad (4).$$

$$\text{Oxalacetate} + \text{DPN-H}_2 \rightarrow \text{Malate} + \text{DPN} \quad (5).$$

$$\text{Isocitrate} + \text{TPN} \rightarrow \text{Oxalosuccinate} + \text{TPN-H}_2 \quad (6).$$

The reactions were carried out in quartz microabsorption cells of 1 cm. depth in the Beckman model DU spectrophotometer; the enzymatic activity was measured by the change in light absorption at 340 mμ resulting from the oxidation or reduction of the pyridine nucleotide. The conditions outlined by Mehler *et al.* (13) were closely adhered to, except for the use of microcells requiring only one-tenth of the volume—viz., 0.3 ml. This resulted in a considerable saving of nucleotide without appreciable sacrifice of accuracy. After several preliminary readings, the reaction was started by the addition of substrate and readings taken at 15-second intervals. In accordance with Mehler *et al.* (13), the enzymatic activity is given in units per milligram of acetone powder, the unit of activity being the amount of enzyme giving a change in log (I_0/I) of 0.01 per minute at room temperature (22°–26°). A blank determination, with substrate omitted, was run simultaneously, and the values, ranging between 0 and 20 units/mg acetone powder, were subtracted from the gross rates to obtain corrected values, which are displayed in Table 3. A considerable range in activities was observed for normal tissues, but in every instance the values for neoplastic tissues were within this range. The general impression one obtains from an over-all survey of Table 3 is that the content of these dehydrogenases in neoplastic tissues is on the low side, but definitely within the range of activities displayed by normal tissues. The data on lactic dehydrogenase are in substantial agreement with those of Meister (14), who used a spectrophotometric method similar to the one employed here, and

TABLE 3

DEHYDROGENASE ASSAYS IN ACETONE POWDER EXTRACTS

The test media were as follows: for lactic and malic dehydrogenases, 0.25 M glycylglycine buffer, pH 7.4, 0.03 ml.; 1.7×10^{-3} M DPN-H_2, 0.02 ml.; 0.04 M pyruvate, 0.02 ml., or 0.01 M oxalacetate, 0.10 ml.; 1:1000 acetone powder extract, 0.04 ml.; and water to a total volume of 0.30 ml. For isocitric dehydrogenase, 0.25 M glycylglycine buffer, pH 7.4, 0.03 ml.; 6.6×10^{-4} M TPN, 0.03 ml.; 0.05 M $MnCl_2$, 0.01 ml.; 1:500 acetone powder extract, 0.10 ml.; and water to 0.30 ml.

Tissue	Dehydrogenase (Units/mg acetone powder) Lactic	Malic	Isocitric
Normal:			
Heart (rat)	320	383	56.0
Liver (mouse)	200	256	10.8
Kidney (rat)	104	173	66.0
Muscle (mouse)	520	330	15.6
Neoplastic:			
Rhabdomyosarcoma (mouse)	160,220	148,208	6.7
Mammary tumor (mouse)	168	380	16.0
Hepatoma (rat)	238	540	12.5–12.8*
Hepatoma (mouse)	108	288	14.8
Ascites (mouse)	165	200	4.8

* Range of three determinations.

those for isocitric dehydrogenase were in accord with previous assays of this enzyme using the Thunberg technic (2).

Oxalacetic carboxylase.—Although this enzyme is not directly involved in the reactions of the citric acid cycle, its assay was included in this study because it undoubtedly plays an important role in the maintenance of the level of oxalacetate and thereby exercises control over the rate of the cycle. Its activity in whole, nondialyzed homogenates was assayed according to the procedure of Vennesland *et al.* (30). The rate of breakdown of oxalacetate was estimated manometrically at pH 4.5 and was corrected for spontaneous breakdown by subtracting the CO_2 evolved in a control flask containing no tissue. A further correction, for endogenous CO_2 production, was applied by subtracting the CO_2 production in the absence of substrate.

TABLE 4

OXALACETIC CARBOXYLASE IN NORMAL AND NEOPLASTIC TISSUE

The test was carried out for 20 minutes at 25° (N_2 gas phase) in conventional Warburg flasks containing the following substances in the designated final concentrations: acetate buffer, pH 4.5, 0.1 M; TPN, 25 μg.; $MnCl_2$ 0.001 M; oxalacetic acid, pH 4.5, 0.02 M; and tissue in a total volume of 3 ml. The rate of spontaneous decarboxylation of oxalacetic acid during the test period (determined from a flask run simultaneously, containing all constituents except tissue) was subtracted from the CO_2 evolved in the complete system. A blank without substrate permitted evaluation of the endogenous CO_2. The reaction rate expressed represents the net μl of CO_2 evolved/milligram of dry tissue/hour.

Tissue	net μl CO_2 evolved / mg dry tissue×hr
Normal:	
Liver (mouse)	3.21
Liver of hepatoma-bearing rat	2.40
Neoplastic:	
Ascites (mouse)	3.48
Hepatoma (rat)	2.45
Rhabdomyosarcoma (mouse)	2.17
Mammary gland adenocarcinoma (mouse)	1.24
Hepatoma (mouse)	0.78

The values given in Table 4 are expressed in μl/mg dry tissue/hour at 25° and are calculated from CO_2 outputs measured for the first 20 minutes. The data leave no doubt concerning the presence of this enzyme in neoplastic tissues in amounts comparable to its content in normal liver cells.

α-Ketoglutaric dehydrogenase.—Although the mechanism of ketoglutarate oxidation is not yet well understood, the reactions involved are probably analogous to those of pyruvate oxidation (7), viz., a thiamine pyrophosphate and DPN-promoted series of reactions leading to formation of CO_2 and a succinic acid-coenzyme A complex. Lacking specific assay procedures for the individual steps, the capacity of neoplastic tissues for carrying out the oxidation of ketoglutarate was tested by measuring the increase in oxygen consumption produced on addition of the keto acid to washed, sedimentable particles of tumors, prepared according to the procedure of Kennedy and Lehninger (8), and fortified by additions of cytochrome c, ATP, DPN, and magnesium ions.[3] The results, as given in Table 5, are reported as Q_{O_2} (μl of oxygen consumed/equivalent of 1 mg dry weight of original tissue/hour). Although α-ketoglutarate oxidation was considerably more rapid in normal tissues, all the tumors displayed the ability to oxidize this acid. In view of the complexity of the system, the significance of these quantitative differences cannot be evaluated, but it can be concluded that tumors can oxidize ketoglutarate.

DISCUSSION

The present study provides definite evidence that neoplastic tissues possess the enzymatic equipment for carrying out reactions of the Krebs cycle. Although the procedures employed were not designed to give rigorous quantitative assays,[4] the results adequately demonstrate that specific enzymatic processes known to be involved in the oxidation of carbohydrates and fatty acids by normal tissues can occur in tumors at rates which are comparable to those of normal tissues. Several enzymes were found to have considerably lower activities in tumors than in normal cells; this was particularly true of aconitase and α-ketoglutaric dehydrogenase. It is possible that these enzymes (or others) constitute "bottle-necks" for the citric

TABLE 5

α-KETOGLUTARATE OXIDATION IN WASHED PARTICLES

The medium contained, in a total volume of 1.6 ml., the following substances in the designated final concentrations: α-ketoglutarate, 7×10^{-3} M; $MgSO_4$, 3×10^{-3} M; fumarate, 7×10^{-5} M; ATP, 2×10^{-3} M; cytochrome c, 4×10^{-6} M; DPN, 1.9×10^{-3} M; phosphate buffer, pH 7.4, 6×10^{-3} M; and homogenate equivalent to 100 mg. of fresh tissue. Experiments were run 1 hour at 38° with air as the gas phase.

Tissue	Net O_2 uptake (μl) (0.3 cc of 1:2 homog)×hr	Net Q_{O_2}
Normal:		
Liver (rat)	152	7.7
Kidney (mouse)	78.7	4.0
Kidney (rat)		11.3
Neoplastic:		
Rhabdomyosarcoma (mouse)	65.1	3.3
Mammary gland adenocarcinoma (mouse)	62.7	3.2
Hepatoma (rat)	46.5	2.4
Hepatoma (mouse)	42.3	2.1

[3] The method used was similar to one described recently by Ackermann (1).

[4] For example, the content of an enzyme in terms of acetone powder may not reflect its concentration in the fresh tissue, because of variations in water and lipid content.

acid cycle, and their low activity may account for some of the phenomena which have led previous investigators to conclude that carbohydrate or pyruvate oxidation was disturbed in neoplastic cells (20). On the other hand, since wide differences in oxygen consumption are not observed between intact normal and neoplastic tissues (31, 35), it seems probable that differences in enzymatic activity are due to various degrees of loss or inactivation during preparation rather than to different concentrations in the intact cell.

Perhaps the most significant observations made in this study were (*a*) that the condensing enzyme is present in neoplastic tissue and (*b*) that malic and isocitric dehydrogenases are present in tumors in amounts comparable to those of normal tissue. The presence of the condensing enzyme definitely establishes the capacity of neoplastic tissues to form citric acid, a matter upon which considerable doubt was cast, particularly by the recent studies of Potter and Busch (19). On the basis of the observation of Buffa and Peters (4) that citrate accumulated in tissues poisoned with fluoroacetate, these investigators fed fluoroacetate to tumor-bearing rats and found that no citrate accumulated in the tumor. They reasoned from these and other results that "the ability of tumor tissues to form citrate is very low in comparison with a wide variety of normal tissues." From the present results it would appear that the nonaccumulation of citrate in fluoroacetate-poisoned neoplastic tissues cannot be due to absence of the condensing enzyme.

The finding that lactic, malic, and isocitric dehydrogenases are present in significant quantities in the neoplasms studied here is of particular interest in view of the low values of malic dehydrogenase for rat tumors reported by Potter (18). We believe that these differences are due primarily to differences in experimental procedure. The method here employed is based directly on measurement of the oxidation or reduction of the coenzyme and is therefore dependent only on the presence of the specific apoenzyme. However, the method used by Potter, viz., measurement of oxygen uptake, requires an intact electron transport system. Although known co-factors were added, there is substantial reason to assume that the concentration of DPN was a limiting factor in the assays of Potter (33, 37). Other evidence may be cited in support of this thesis. Rhian and Potter (24), again using a manometric method, reported that DPN-cytochrome c reductase was low in tumors, whereas Hogeboom and Schneider, using a spectrophotometric method, found a *higher* content of this enzyme in neoplastic than in normal tissues (6). Possibly some of the low values for lactic and malic dehydrogenases reported in tumors by Lenta and Riehl, using the Thunberg technic (10), may also have been due to lack of an essential electron transport factor.

SUMMARY

A series of transplanted mouse and rat tumors was assayed for their content of citric acid cycle and related enzymes, and the results were compared with a representative series of normal tissues. Lactic, malic, and isocitric dehydrogenases, fumarase, oxalacetic carboxylase, and the "condensing" enzyme were found present in tumors in amounts comparable to those in normal tissues. Aconitase and α-ketoglutarate dehydrogenase were present in tumors but to lower extents than in normal tissues. The results warrant the conclusion that neoplastic tissues possess the enzymatic equipment for the citric acid cycle.

REFERENCES

1. Ackermann, W. W. α-Ketoglutaric Acid Oxidase; An Assay Procedure. J. Biol. Chem., **184**:557–62, 1950.
2. Adler, E.; Euler, H. v.; Günther, G.; and Plass, M. Isocitric Dehydrogenase and Glutamic Acid Synthesis in Animal Tissues. Biochem. J., **33**:1028–45, 1939.
3. Buchanan, J. M., and Anfinsen, C. B. Partial Purification of Aconitase, J. Biol. Chem., **180**:47–54, 1949.
4. Buffa, P., and Peters, R. A. Formation of Citrate *in vivo* Induced by Fluoroacetate Poisoning. Nature, **163**:914, 1949.
5. Greville, G. D. The Effect of Calcium Ion on Tissue Respiration, with a Note on the Estimation of Oxaloacetic Acid. Biochem. J., **33**:718–22, 1939.
6. Hogeboom, G. H., and Schneider, W. C. Intracellular Distribution of Enzymes. VIII. The Distribution of Diphosphopyridine Nucleotide-Cytochrome c Reductase in Normal Mouse Liver and Mouse Hepatoma. J. Nat. Cancer Inst., **10**:983–87, 1950.
7. Korkes, S.; Stern, J. R.; Gunsalus, I. C.; and Ochoa, S. Enzymatic Synthesis of Citrate from Pyruvate and Oxalacetate. Nature, **166**:439–40, 1950.
8. Kennedy, E. P., and Lehninger, A. L. The Requirements of the Fatty Acid Oxidase Complex of Rat Liver. J. Biol. Chem., **173**:753–71, 1948.
9. Krampitz, L. O., and Werkman, C. H. The Enzymatic Decarboxylation of Oxalacetate. Biochem. J., **35**:595–602, 1941.
10. Lenta, Sister M. P., and Riehl, Sister M. A. Dehydrogenase Studies of Tissue from Normal and Tumor-bearing Mice. II. Lactic and Malic Dehydrogenase. Cancer Research, **9**:47–50, 1949.
11. LePage, G. A., and Mueller, G. C. Preparation of Triphosphopyridine Nucleotide. J. Biol. Chem., **180**:975–84, 1949.
12. Lynen, F., and Reichart, E. Zur chemischen Struktur der "Aktivierten Essigsäure." Angewandte Chem., **63**:47–48, 1951.
13. Mehler, A. H.; Kornberg, A.; Grisolia, S.; and Ochoa, S. The Enzymatic Mechanism of Oxidation Reductions between Malate or Isocitrate and Pyruvate. J. Biol. Chem., **174**:961–77, 1948.
14. Meister, A. Lactic Dehydrogenase Activity of Certain

Tumors and Normal Tissues. J. Nat. Cancer Inst., **10**: 1263–71, 1950.

15. NATELSON, S.; PINCUS, J. B.; and LUGOVOY, J. K. Microestimation of Citric Acid; A New Colorimetric Reaction for Pentabromoacetone. J. Biol. Chem., **175**:745–50, 1948.
16. NOVELLI, G. D., and LIPMANN, F. The Catalytic Function of Coenzyme A in Citric Acid Synthesis. J. Biol. Chem., **182**:213–28, 1950.
17. OHLMEYER, P. Preparation of Pure Dihydrocozymase. Biochem. Ztschr. **297**:66–69, 1938.
18. POTTER, V. R. The Assay of Animal Tissues for Respiratory Enzymes. V. The Malic Dehydrogenase System. J. Biol. Chem., **165**:311–24, 1946.
19. POTTER, V. R., and BUSCH, H. Citric Acid Content of Normal and Tumor Tissues *in Vivo* Following Injection of Fluoracetate. Cancer Research, **10**:353–56, 1950.
20. POTTER, V. R.; LEPAGE, G. A.; and KLUG, H. L. The Assay of Animal Tissues for Respiratory Enzymes. VII. Oxalacetate Acid Oxidation and the Coupled Phosphorylations in Isotonic Homogenates. J. Biol. Chem., **175**:619–34, 1948.
21. RACKER, E. Spectrophotometric Measurements of the Enzymatic Formation of Fumaric and cis-Aconitic Acids. Biochim. & Biophys. Acta, **4**:211–14, 1950.
22. ———. Crystalline Alcohol Dehydrogenase from Baker's Yeast. J. Biol. Chem., **184**:313–19, 1950.
23. RHIAN, M., and POTTER, V. R. The DPN-Cytochrome Reductase Content of Cancer Tissue. Cancer Research, **7**:714, 1947.
24. SCHNEIDER, W. C., and HOGEBOOM, G. H. Intracellular Distribution of Enzymes. VI. The Distribution of Succinoxidase and Cytochrome Oxidase Activities in Normal Mouse Liver and in Mouse Hepatoma. J. Nat. Cancer Inst., **10**:969–75, 1950.
25. SCHNEIDER, W. C., and POTTER, V. R. Biocatalysts in Cancer Tissue. III. Succinic Dehydrogenase and Cytochrome Oxidase. Cancer Research, **3**:353–57, 1943.
26. STADTMAN, E. R. Coenzyme A-dependent Transacetylation and Transphosphorylation. Fed. Proc., **9**:233, 1950.
27. STERN, J. R., and OCHOA, S. Enzymatic Synthesis of Citric Acid by Condensation of Acetate and Oxalacetate. J. Biol. Chem., **179**:491–92, 1949.
28. ———. Enzymatic Synthesis of Citric Acid. I. Synthesis with Soluble Enzymes. *Ibid.*, **191**:161–72, 1951.
29. STERN, J. R.; SHAPIRO, B.; and OCHOA, S. Synthesis and Breakdown of Citric Acid with Crystalline Condensing Enzyme. Nature, **166**:403–4, 1950.
30. VENNESLAND, B.; EVANS, E. A.; and ALTMAN, K. I. The Effects of Triphosphopyridine Nucleotide and of Adenosine Triphosphate on Pigeon Liver Oxalacetic Carboxylase. J. Biol. Chem., **171**:675–86, 1947.
31. WARBURG, O. Metabolism of Tumours. London: Constable & Co., 1930.
32. WARBURG, O., and CHRISTIAN, W. A New Oxidation Enzyme and Its Adsorption Spectrum. Biochem. Ztschr. **254**:438–58, 1932.
33. WEINHOUSE, S. Studies on the Fate of Isotopically Labeled Metabolites in the Oxidative Metabolism of Tumors. Cancer Research, **11**:585–91, 1951.
34. WEINHOUSE, S.; MILLINGTON, R. H.; and WENNER, C. E. Occurrence of the Citric Acid Cycle in Tumors. J. Am. Chem. Soc., **72**:4332, 1950.
35. ———. Metabolism of Neoplastic Tissue. I. The Oxidation of Carbohydrates and Fatty Acids in Transplanted Tumors. Cancer Research, **11**:845–50, 1951.
36. WENNER, C. E.; SPIRTES, M. A.; and WEINHOUSE, S. Enzymes of the Citric Acid Cycle in Tumors. J. Am. Chem. Soc., **72**:4333, 1950.
37. ———. Activation of Pyruvate Oxidation in Tumor Mitochondria by Diphosphopyridine Nucleotide. Proc. Soc. Exper. Biol. & Med. (in press).
38. WISLICENUS, W., and WALDMÜLLER, M. Oxalosuccinic Ester. Ber., **44**:1565–73, 1911.

The Synergistic Action of Mixtures of Certain Hepatic Carcinogens*

J. C. MacDonald, E. C. Miller, J. A. Miller, and H. P. Rusch

(*McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison 6, Wis.*)

INTRODUCTION

Both 2-acetylaminofluorene (AAF) and certain aminoazo dyes structurally related to 4-dimethylaminoazobenzene (DAB) induce high incidences of liver tumors when fed to rats. In each case the neoplasms have been classified as both hepatomas and cholangiomas (cf. 4, 6, 17, 18). The ingestion of compounds of either type causes marked decreases in the pentosenucleic acid, protein, and riboflavin contents of certain fractions of the liver cells, and in both cases the greatest alterations occur in the composition of the large granules or mitochondria[1] (19). The tumor cells induced by either type of compound contain similar amounts of these constituents, and the levels are lower than those found in the damaged liver cells (7, 8, 20). The desoxypentosenucleic acid content of the average tumor cell induced by either type of agent is essentially the same as that for an average liver cell. However, both types of liver tumors have a higher nuclear density than liver and thus contain more desoxypentosenucleic acid per gram of tissue than does liver.[1]

These similarities in the action of the two types of compounds suggested that their carcinogenic processes might also have some features in common. As one approach to this problem, experiments were carried out to determine whether minimal doses of two carcinogens would give a synergistic response. Such a synergism has been found when low levels of 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB) and 2-acetylaminofluorene were fed together. These results appear to be consistent with, but not proof of, the hypothesis that there is a phase in the carcinogenic process which is common to both agents.

* This work was supported in part by grants from the National Cancer Institute, Public Health Service, and the American Cancer Society upon recommendation by the National Research Council.

[1] A. K. Laird and E. C. Miller, unpublished data.

Received for publication September 25, 1951.

METHODS

Male albino rats,[2] weighing 180–220 gm. (Series A), 195–250 gm. (Series B), or 160–200 gm. (Series C and D) were housed in screen-bottom cages in groups of four with feed and water available ad libitum.

The basal diet for Series A and B was composed of ground yellow corn, 68; linseed oil meal, 16; powdered skim milk, 12; alfalfa leaf meal, 2; cod liver oil, 1; iodized salt, 0.5; and tricalcium phosphate, 0.5. The diets containing the carcinogens were made by mixing 990 gm. of this diet with 10 gm. of a glucose triturate of the carcinogen, and each diet was fed to 15–20 rats. This crude diet was selected, since it permits more rapid liver tumor formation with AAF (13) than any purified diet reported so far (7).

In Series A (Table 1) Groups 1–3 were fed diets containing either 0.018 per cent of AAF, 0.020 per cent of 3′-Me-DAB, or both of these compounds for 180 days. Groups 4–7 were fed diets containing either 0.036 per cent of AAF or 0.040 per cent of 3′-Me-DAB for 90 days, followed by rations containing either the other carcinogen or the basal diet for another 90 days. Groups 8–10 (Series B) were duplicates of Groups 1–3 (Series A), except that the carcinogens were fed for only 130 days. The ration for Group 11 had 0.025 per cent of 3-acetylaminodibenzothiophene (ABT) and 0.020 per cent of 3′-Me-DAB, while Group 12 was given 0.064 per cent of 4′-methyl-4-dimethylaminoazobenzene (4′-Me-DAB) in addition to 0.018 per cent of AAF. Groups 13 and 14 received diets to which 0.025 per cent of 3-ABT and 0.064 per cent of 4′-Me-DAB, respectively, had been added. The rats in the latter groups were fed the carcinogens for 130 days.

At the end of the feeding periods indicated, the livers of all the surviving rats were examined by laparotomy, and the rats were then fed the basal

[2] Obtained from the Holtzman-Rolfsmeyer Rat Co., Madison, Wis.

diet for an additional 95 days to allow latent tumors to develop. Tumor-bearing rats were killed throughout the experiment when tumors were large or death appeared imminent, and the surviving rats were killed at the end for a final tumor count. In the case of tumor-bearing rats at least one tumor from the liver or the intestinal epithelium and all other tumors (except those arising from the ear duct) or questionable areas were fixed in Mossman's modification of Laudowsky's formalin, alcohol, and acetic acid mixture (9), sectioned, stained with hematoxylin and eosin, and examined microscopically. The tumors originating from the ear duct were not examined microscopically, since this type of tumor had been studied earlier (5, 10).

In Series C four groups of twelve rats each were fed diets containing 0.030 or 0.060 per cent of 4-dimethylaminoazobenzene (DAB), 0.064 per cent of 4′-Me-DAB, or 0.030 per cent of DAB plus 0.032 per cent of 4′-Me-DAB. The basal diet for this series was composed of crude casein, 12; Vitab,[3] 2; salts, 4; glucose, 77; corn oil, 5; and riboflavin to a total content of 1.0 mg/kg; the dyes were added to the diets in corn oil solution. After the dye-containing diets had been fed for 130 days, the liver of each rat was examined by laparotomy, and the rats were then fed the basal diet for 60 days before the final tumor count. Series D was a duplicate of Series C, except that the diets contained 2.0 mg. of riboflavin/kg, and the dye-containing diets were fed for 150 days.

The aminoazo dyes used in these experiments were synthesized by the general procedure for the preparation of N,N-dialkyl-substituted dyes (15) and recrystallized from benzene-petroleum ether mixtures. For the preparation of AAF, 2-aminofluorene, made from fluorene as described by Kuhn (12), was dissolved in a large volume of benzene and acetylated by the addition of a 10 per cent excess of acetic anhydride in benzene solution. The reaction mixture was boiled under reflux for 1 hour, and the AAF which crystallized out was washed repeatedly with benzene. ABT (3) was generously furnished by Dr. R. B. Sandin of the University of Alberta.

TABLE 1

TUMOR INCIDENCES IN RATS FED VARIOUS COMBINATIONS OF CERTAIN HEPATIC CARCINOGENS

Group no.	Carcinogens* fed for the period		Survival†	No. rats with gross cirrhosis:		No. rats bearing tumors in the				Negative survivors
				Mild	Moderate	Liver		Ear duct	Small intestine	
Series A:	(0–90 days)	(90–180 days)	(180 days)	(180 days)		(180 days)	(270 days)	(270 days)	(270 days)	(270 days)
1	0.018 per cent AAF+0.020 per cent 3′-Me-DAB	same	16/16	9	7	10	16	3	0	0
2	0.018 per cent AAF	same	16/16	10	2	1	12	3	1	1‡
3	0.020 per cent 3′-Me-DAB	same	16/16	0	0	0	0	0	0	16
4	0.036 per cent AAF	0.040 per cent 3′-Me-DAB	16/16	9	7	10	14	3	4	0
5	0.040 per cent 3′-Me-DAB	0.036 per cent AAF	15/16	10	5	4	12	0	0	1‡
6	0.036 per cent AAF	none	16/16	7	4	9	13	3	7	0
7	0.040 per cent 3′-Me-DAB	none	16/16	0	0	1	2	0	0	14
Series B:	(0–130 days)		(130 days)	(130 days)		(130 days)	(220 days)	(220 days)	(220 days)	(220 days)
8	0.018 per cent AAF+0.020 per cent 3′-Me-DAB		20/20	6	10	9	19	2	2	0
9	0.018 per cent AAF		20/20	9	0	0	2	0	0	16
10	0.020 per cent 3′-Me-DAB		19/20	0	0	0	0	0	0	17
11	0.025 per cent ABT+0.020 per cent 3′-Me-DAB		15/15	0	0	0	1	8	1	6
12	0.018 per cent AAF+0.064 per cent 4′-Me-DAB		14/15	4	1	0	3	0	0	8‡
13	0.025 per cent ABT		14/15	0	0	0	0	3	3	8
14	0.064 per cent 4′-Me-DAB		15/15	0	0	0	0	0	0	7

* AAF = 2-acetylaminofluorene; ABT = 3-acetylaminodibenzothiophene; 3′-Me-DAB = 3′-methyl-4-dimethylaminoazobenzene; 4′-Me-DAB = 4′-methyl4-dimethylaminoazobenzene.

† Survival = number of rats alive at the end of the compound-feeding period over the number started.

‡ Other tumors found in this series included a myxofibroma (Group 5), a benign cystadenoma (Group 9), a fibrosarcoma (Group 13), and a mammary adenocarcinoma (Group 2).

RESULTS

Combined feeding of 3′-methyl-4-dimethylaminoazobenzene and 2-acetylaminofluorene.—The data on the survival and tumor incidences for the rats fed AAF and 3′-Me-DAB in the grain diet are detailed in Table 1. Most of the rats gained 120–200 gm. during the period in which the compounds were fed, and in both series the survival of the rats to the end of the carcinogen-feeding periods was excellent. In general, the survival of the nontumor-bearing rats to the end of the experiment was also good, but a high incidence of fatal respiratory infection at about 175 days decreased the number of

[3] Obtained from the National Oil Products Co., Harrison, New Jersey.

survivors in the group fed 4′-Me-DAB (Group 14). Most of the rats fed AAF had a mild to moderate degree of gross hepatic cirrhosis, while those not fed AAF generally had little or no gross evidence of liver damage.

A striking synergistic action in the induction of liver tumors was observed when low levels of AAF and 3′-Me-DAB were fed simultaneously. At 180 days hepatic tumors were found in 62 per cent of the rats fed the diet containing both AAF and 3′-Me-DAB (Group 1), while only 6 and 0 per cent of those fed either AAF or 3′-Me-DAB alone had liver tumors at this time (Groups 2 and 3). After 95 days on the basal diet the tumor incidences were 100, 75, and 0 per cent for those fed the mixture, AAF, or 3′-Me-DAB, respectively. When this experiment was repeated with a carcinogen-feeding period of only 130 days, the synergistic action of the two compounds was even more definite. Thus, 45 per cent of the rats fed the mixture of AAF and 3′-Me-DAB had liver tumors by 130 days, while none could be detected in the livers of animals fed either compound alone (Groups 8–10). After the basal diet had been fed for 95 days, the tumor incidence rose to 95 per cent for the group fed both carcinogens, while liver tumors were found in only 10 and 0 per cent of the rats fed AAF or 3′-Me-DAB alone.

When 0.036 per cent of AAF was fed for the first 90 days and 0.040 per cent of 3′-Me-DAB was given the second 90 days, 88 per cent of the rats developed liver tumors (Group 4). Eighty per cent of the animals fed these carcinogens in the reverse sequence had hepatic tumors at the end of the experiment (Group 5). However, since 81 per cent of the control rats fed this level of AAF for 90 days and then maintained on the basal diet also developed this type of tumor, no conclusions regarding the additive action of these compounds when fed in sequence are possible. Only two of the sixteen rats fed 0.040 per cent of 3′-Me-DAB in this diet for 90 days had tumors by the end of the experiment.

The synergistic action of 3′-Me-DAB and AAF in the induction of liver tumors raised the question of whether a weak carcinogen of either series might augment the activity of a strong carcinogen of the other series. The compounds chosen for this experiment were 4′-Me-DAB, which is less than one-twelfth as active as 3′-Me-DAB in the induction of hepatic tumors (14), and ABT (a sulfur analog of AAF), which is as active as AAF in the induction of tumors of the ear duct, mammary gland, and small intestinal epithelium[4] but does not cause liver tumors (13). When 4′-Me-DAB and AAF were fed simultaneously, no evidence of synergism was obtained, since the tumor incidence was essentially the same as that observed when the same level of AAF was fed alone (Groups 9 and 12). Similarly, when 0.025 per cent of ABT and 0.020 per cent of 3′-Me-DAB were fed together, only one of 15 rats developed liver tumors, and no hepatic tumors were found in the animals receiving either of these compounds alone (Groups 10, 11, and 13).

Regardless of the compound or mixture of carcinogens fed, most of the hepatic neoplasms found in the present experiment appeared to be derived from the parenchymatous cells. Thus, in the various groups 77–93 per cent of the liver tumors were classified as hepatomas, 7–24 per cent as cholangiomas, and 8 per cent as adenocarcinomas (6, 17). Ten to 20 per cent of the tumors had elements of both hepatomas and cholangiomas, and these tumors were classified according to the predominant cell type in the sections studied. No characteristic differences were observed between the tumors induced solely by AAF or by a mixture of AAF and 3′-Me-DAB. Only one neoplasm induced solely by 3′-Me-DAB was available for study.

As in other experiments (2, 13), appreciable numbers of squamous-cell carcinomas arising from the ear duct and adenocarcinomas of the small intestine[4] were found in the rats fed either AAF or ABT. While the simultaneous ingestion of 3′-Me-DAB and AAF had little or no effect on the rate of development of these tumors, the administration of 3′-Me-DAB with ABT appeared to augment the induction of the tumors arising from the ear duct. Thus, eight of the rats fed 3′-Me-DAB and ABT together and only three of those fed ABT alone had tumors of this type at the end of the experiment. Other less frequently occurring tumors included a benign cystadenoma near the vertebral column of a rat in Group 9 fed AAF and a fibrosarcoma in the same region of a rat from Group 13 which ingested ABT. A myxofibroma was found under the leg of an animal from Group 5 fed 3′-Me-DAB and AAF in sequence, and an adenocarcinoma of the mammary gland developed in a rat from Group 2 given AAF.

Combined feeding of 4-dimethylaminoazobenzene and 4′-methyl-4-dimethylaminoazobenzene.—Feeding the very weak hepatic carcinogen 4′-Me-DAB simultaneously with a minimal carcinogenic dose of the more active dye DAB markedly stimulated the induction of liver tumors (Table 2). Thus, in Series C only 16 per cent of the rats fed 0.030 per

[4] The occurrence of this type of tumor was not noted in our previous paper (13) since only a casual examination of the intestines was made. It is necessary to strip the intestines from the mesentery to expose adequately these tumors.

cent of DAB had developed liver tumors by 190 days, while 67 per cent of those fed 0.030 per cent of DAB plus 0.032 per cent of 4′-Me-DAB developed tumors in the same period. None of the rats fed 4′-Me-DAB at twice this level developed tumors, and other experiments (14, 16) have shown that 0.064 per cent of 4′-Me-DAB must be fed to rats for 8–12 months to induce even a low incidence of hepatic tumors. However, extra DAB was a more effective supplement than 4′-Me-DAB, since all the rats fed 0.060 per cent of DAB developed liver tumors and the induction period was considerably shorter.

In Series D, in which the rate of tumor induction was slower (probably because of the higher level of dietary riboflavin), none of the rats fed 0.030 per cent of DAB or 0.064 per cent of 4′-Me-DAB developed liver tumors. However, 21 per cent of the animals fed 0.030 per cent of DAB and 0.032 per cent of 4′-Me-DAB together and 87 per cent of those given 0.060 per cent of DAB had tumors by the end of the experiment.

TABLE 2

INCIDENCE OF HEPATIC TUMORS IN RATS FED 4-DIMETHYLAMINOAZOBENZENE AND 4′-METHYL-4-DIMETHYLAMINOAZOBENZENE

Group	Diet	Survival*	Gross cirrhosis	Hepatic tumors		Negative survivors
Series C:		(130 days)	(130 days)	(130 days)	(190 days)	(190 days)
1	0.060 per cent DAB†	12/12	Moderate-Severe	11	12	0
2	0.030 per cent DAB	12/12	None-Mild	1	2	10
3	0.030 per cent DAB+0.032 per cent 4′-Me-DAB†	9/12	Mild-Moderate	2	6	3
4	0.064 per cent 4′-Me-DAB	12/12	None	0	0	12
Series D:		(150 days)	(150 days)	(150 days)	(210 days)	(210 days)
5	0.060 per cent DAB	15/16	Mild-Moderate	4	13	0
6	0.030 per cent DAB	13/16	None-Mild	0	0	8
7	0.030 per cent DAB+0.032 per cent 4′-Me-DAB	14/15	None-Mild	0	3	11
8	0.064 per cent 4′-Me-DAB	17/17	None	0	0	12

* Survival = number of rats alive at end of dye-feeding over number started.
† DAB = 4-dimethylaminoazobenzene; 4′-Me-DAB = 4′-Methyl-4-dimethylaminoazobenzene.

DISCUSSION

The data clearly demonstrate that the simultaneous administration of AAF and 3′-Me-DAB or DAB and 4′-Me-DAB resulted in a higher incidence of liver tumors than the sum of the incidences obtained when the same levels of each of the compounds were fed singly. However, the mechanism through which these pairs of compounds produced a synergistic response is difficult to ascertain. One approach is to assume, as a working hypothesis, that tumor induction by these compounds is a multistage process and that the over-all rate is determined largely by the rate at the slowest step. Then the carcinogenic response should be no greater than the sum of the responses induced by each compound alone, if the neoplastic processes induced by each agent were entirely independent of one another or if the processes were similar and limited at the same step. On the other hand, a synergistic response would be expected if the processes possessed one common step which was limiting for only one of the two carcinogens. Synergism would also be expected if the carcinogenic processes had two or more steps in common and each had a different rate-limiting step.

However, other factors must also be considered. Data of this type might also result from altered rates or pathways of metabolism following simultaneous administration of the two compounds. For instance, the rat may be able to maintain a higher effective level of one or both carcinogens in the liver cells when the compounds are administered together than would be observed if the metabolism of each compound were unaffected by the presence of the other. Again, the role of irritation in stimulating the growth of latent tumor cells in the skin of mice following minimal applications of the carcinogenic hydrocarbons (1, 11) suggests that under some conditions regeneration of damaged areas in the liver might favor the growth of latent hepatic tumor cells. Thus, 3′-Me-DAB might act as a co-carcinogen for AAF, while AAF was also a co-carcinogen for 3′-Me-DAB.

Quite different results were observed by Richardson and Cunningham (21) when 20-methylcholanthrene was administered intravaginally to rats receiving 3′-Me-DAB in the diet. Under these conditions there was a marked reduction in the incidence of hepatic tumors and the degree of liver damage as compared to that observed when 3′-Me-DAB was administered alone.

SUMMARY

1. When 2-acetylaminofluorene and 3′-methyl-4-dimethylaminoazobenzene were fed simultaneously in a grain diet to male rats, the incidence of hepatic tumors was greater than the sum of the tumor incidences obtained when the carcinogens were fed singly. No synergistic effect on liver tumor induction was observed when 3′-methyl-4-dimethylaminoazobenzene and 3-acetylaminodibenzothiophene or 4′-methyl-4-dimethylaminoazobenzene and 2-acetylaminofluorene were fed together.

2. The incidence of tumors arising from the ear duct was greater in rats fed both 3′-methyl-4-dimethylaminoazobenzene and 3-acetylaminodibenzothiophene than in animals fed only the latter compound. 3′-Methyl-4-dimethylaminoazobenzene did not augment the induction of ear duct tumors by 2-acetylaminofluorene.

3. The simultaneous administration of 0.032 per cent of 4′-methyl-4-dimethylaminoazobenzene and 0.030 per cent of 4-dimethylaminoazobenzene was a more effective carcinogenic stimulus for the liver of male rats than either 0.030 per cent of 4-dimethylaminoazobenzene or 0.064 per cent of 4′-methyl-4-dimethylaminoazobenzene alone; it was a less effective dose than 0.060 per cent of 4-dimethylaminoazobenzene.

4. 2-Acetylaminofluorene and 3-acetylaminodibenzothiophene also induced tumors in the small intestinal epithelium of 10–35 per cent of the rats fed these compounds.

REFERENCES

1. Berenblum, I. The Mechanism of Carcinogenesis. A Study of the Significance of Cocarcinogenic Action and Related Phenomena. Cancer Research, **1**:807–14, 1941.
2. Bielschowsky, F. Comparison of the Tumours Produced by 2-Acetylaminofluorene in Piebald and Wistar Rats. Brit. J. Exper. Path., **27**:135–39, 1946.
3. Brown, R. K.; Christiansen, R. G.; and Sandin, R. B. Some Derivatives of Dibenzothiophene. J. Am. Chem. Soc., **70**:129–31, 1944.
4. Cantarow, A.; Paschkis, K. E.; Stasney, J.; and Rothenberg, M. S. The Influence of Sex Hormones upon the Hepatic Lesions Produced by 2-Acetaminofluorene. Cancer Research, **6**:610–16, 1946.
5. Cox, A. J., Jr.; Wilson, R. H.; and DeEds, F. The Carcinogenic Activity of 2-Acetaminofluorene. Characteristics of the Lesions in Albino Rats. Cancer Research, **7**:647–57, 1947.
6. Edwards, J. E., and White, J. Pathologic Changes, with Special Reference to Pigmentation and Classification of Hepatic Tumors in Rats Fed *p*-Dimethylaminoazobenzene (Butter Yellow). J. Nat. Cancer Inst., **2**:157–83, 1941.
7. Griffin, A. C.; Cook, H.; and Cunningham, L. Tissue Proteins and Carcinogenesis. III. Precancerous Changes in the Liver and Serum Proteins of Rats Fed Acetylaminofluorene. Arch. Biochem., **24**:190–98, 1949.
8. Griffin, A. C.; Nye, W. N.; Noda, L.; and Luck, J. M. Tissue Proteins and Carcinogenesis. I. The Effect of Carcinogenic Azo Dyes on Liver Proteins. J. Biol. Chem., **176**: 1225–35, 1948.
9. Guyer, M. F. Animal Micrology, p. 239. 4th ed. Chicago: University of Chicago Press, 1941.
10. Harris, P. N. Production of Tumors in Rats by 2-Aminofluorene and 2-Acetylaminofluorene. Failure of Liver Extract and of Dietary Protein Level To Influence Liver Tumor Production. Cancer Research, **7**:88–94, 1947.
11. Kline, B. E., and Rusch, H. P. Some Factors That Influence the Growth of Neoplastic Cells. Cancer Research, **4**:762–67, 1944.
12. Kuhn, W. E. 2-Nitrofluorene and 2-Aminofluorene. In Blatt, A. H. (ed.), Organic Synthesis, Coll. Vol. **2**:447–48. New York: John Wiley & Sons, 1943.
13. Miller, E. C.; Miller, J. A.; Sandin, R. B.; and Brown, R. K. The Carcinogenic Activities of Certain Analogues of 2-Acetylaminofluorene in the Rat. Cancer Research, **9**: 504–9, 1949.
14. Miller, J. A., and Baumann, C. A. The Carcinogenicity of Certain Azo Dyes Related to *p*-Dimethylaminoazobenzene. Cancer Research, **5**:227–34, 1945.
15. Miller, J. A., and Miller, E. C. The Carcinogenicity of Certain Derivatives of *p*-Dimethylaminoazobenzene in the Rat. J. Exper. Med., **87**:139–56, 1948.
16. Nagao, N. Über die Fütterungsversuche mit 4′-Methyl-4-N-Dimethylaminoazobenzol und weiteren Polymethylderivaten bei weissen Ratten. Gann, **35**:8–20, 1941.
17. Opie, E. L. The Pathogenesis of Tumors of the Liver Produced by Butter Yellow. J. Exper. Med., **80**:231–46, 1944.
18. Orr, J. W. The Histology of the Rat's Liver during the Course of Carcinogenesis by Butter-Yellow (*p*-Dimethylaminoazobenzene). J. Path. & Bact., **50**:393–408, 1940.
19. Price, J. M.; Miller, E. C.; Miller, J. A.; and Weber, G. M. Studies on the Intracellular Composition of Livers from Rats Fed Various Aminoazo Dyes. II. 3′-Methyl-, 2′-Methyl-, and 2-Methyl-4-dimethylaminoazobenzene, 3-Methyl-4-monomethylaminoazobenzene, and 4′-Fluoro-4-dimethylaminoazobenzene. Cancer Research, **10**:18–27, 1950.
20. Price, J. M.; Miller, J. A.; Miller, E. C.; and Weber, G. M. Studies on the Intracellular Composition of Liver and Liver Tumor from Rats Fed 4-Dimethylaminoazobenzene. Cancer Research, **9**:96–102, 1949.
21. Richardson, H. L., and Cunningham, L. The Inhibitory Action of Methylcholanthrene on Rats Fed the Azo Dye 3′-Methyl-4-dimethylaminoazobenzene. Cancer Research, **11**:274, 1951.

On the Induction of Malignant Tumors in Pigeons by a Chicken Sarcoma Virus after Previous Adaptation of the Virus to Ducks*

PAULO R. F. BORGES† AND F. DURAN-REYNALS

(Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and the Department of Microbiology, Yale University School of Medicine, New Haven, Conn.)

It was previously reported (5) that cells from duck variants of the Rous sarcoma were effective in inducing malignant growths in adult pigeons inoculated in the breast; this is in contrast to results obtained with the original chicken tumor (2).

The present study is concerned with the effect of filtrates from one of the duck variants, and also with characteristic features of the pigeon tumors—induced by either cells or filtrates—which are associated with a special state, *masking*, of the causative virus.

MATERIALS AND METHODS

The pigeons employed were of the varieties commonly found in the markets; the chickens and ducks were of the Plymouth Rock and Pekin breeds, respectively.

The duck tumor, variant 14 (d) 7 of the Rous sarcoma, was obtained by injecting a newly hatched duck with a cell suspension of the chicken tumor grown in a host 14 months old (4). It is the most malignant of all the variants of the Rous sarcoma, and, in contrast to the behavior of other variants (1), the causative virus kept its pathogenic power for adult chickens despite its adaptation to ducks. In both chicks and ducklings the virus induces rapidly growing tumors which are frequently followed by widespread metastases and hemorrhagic lesions (4, 7).

Cell suspensions from this tumor consisted of 1 part of the tumor tissue passed through a mincer and 4 parts of saline solution. Filtrates were made of 1 part of ground tumor and 19 of saline solution, the resulting extract being passed through a Berkefeld "N" filter.

* This investigation was aided by grants from the Jane Coffin Childs Memorial Fund for Medical Research, the American Cancer Society, and the National Cancer Institute, National Institutes of Health, Public Health Service.

† Fellow of the Finney-Howell Research Foundation.

Received for publication September 28, 1951.

Both cell suspensions and filtrates were injected into the breast of pigeons, ducklings, and chicks at doses of 1 or 2 cc. Filtrates were sometimes injected into the veins of ducklings and chicks. The same methods were followed when tumor tissue or bone marrow from tumor-bearing pigeons was used for further passages. The age of the birds at the time of injection will be indicated in the corresponding sections.

For histological studies, hematoxylin and eosin stain was routinely used. Special staining or impregnation methods were performed for elastic, collagenous, or reticular fibers, for iron-containing pigments, and also for the detection of acid-fast or ordinary bacteria and fungi. Blood smears were stained with the May Grünwald and Giemsa solutions.

Transmissibility of duck sarcomas to pigeons by means of filtrates and cell suspensions.—The results of the inoculation of pigeons of different ages with these tumor materials are given in Table 1.

It is clear from the data that cell suspensions induced tumors in all the twelve pigeons which received injections, while filtrates were grossly effective in only nine of seventeen pigeons. The tumors regressed in five of the pigeons of the former group and in six of the latter group. However, the incidence of regression would have probably been higher in both groups if the tumor-bearing pigeons had not been killed for passage purposes, as was done in all but three cases in which the pigeons died with tumors.

Growth was faster after injection of cells than after that of filtrates, in the former case tumors being noticeable as early as 1 week after inoculation, as compared to 2 or 3 weeks in the latter case. In several birds the tumors attained sizes of 16 sq. cm. after intervals of as long as 5 months. However, regression of tumors of this large size was observed. The growths were soft, infiltrating, and often showed necrotic areas.

Metastases, in the liver and ribs, were observed only in three pigeons; two of them had been injected with cells and the other with filtrate. Hemorrhagic lesions, which so frequently develop in chicks and ducklings injected with tumor viruses (1, 2, 4, 6), were never observed in the pigeons.

Data concerning the transmissibility of the pigeon tumors are given in Table 2. In experiments 2, 3, 5, 7, 8, and 9 the tumors were induced by cells of the duck variant, whereas in experiments 1, 4, 6, and 10 they were induced by filtrates. In this second passage the age of the pigeons varied from 6 weeks to 5 months, and that of the ducklings and chicks from 5 to 10 days. Half the number of the ducklings and chicks was injected with 1 cc. of filtrate in the vein instead of the breast.

TABLE 1

EFFECTIVENESS OF CELLS AND FILTRATES FROM A DUCK VARIANT OF THE ROUS SARCOMA IN INDUCING TUMORS IN PIGEONS

Exp.	Tumor material injected*	No. of pigeons	Age of pigeons	Results: Progressive growth	Growth and regression	No growth
1	C.S.	1	7 days		1	
2	F.	3	7 days	1	1	1
3	C.S.	2	2 wks.	1	1	
4	F.	3	2 wks.		2	1
5	C.S.	2	5 wks.	1	1	
6	F.	3	5 wks.	1	2	
7	C.S.	2	6 mos.		2	
8	F.	3	6 mos.	1	1	1
9	C.S.	5	1–2 yrs.	5		
10	F.	5	1–2 yrs.			5

* C.S. = cell suspension; F. = filtrate.

From the data in the table and some complementary observations the following are clear:

1. Filtrates from the pigeon tumors were inactive in other pigeons, and also in ducklings and chicks.

2. Cell suspensions from the ten pigeon tumors tested were equally inactive in inducing steadily growing tumors in other pigeons. However, temporary growth was obtained, at least in some cases, as shown by the frequent development of nodules in the injected site, and by the fact that in one bird that was killed a nodule of that sort proved to be a tumor. Other lesions of an inflammatory-like character were sometimes found in the breast muscle of the inoculated pigeons. These lesions will be described later.

3. Cell suspensions from four of the five pigeon tumors tested induced, in chicks and ducklings, steadily growing tumors which were frequently followed by widespread metastases and hemorrhagic lesions. Filtrates from one duckling tumor and from one chick tumor proved to be effective on other chicks and ducklings. The only pigeon tumor, in experiment 9, that failed to grow in chicks and ducklings was a tumor 10 weeks old in a host 20 months old.

Microscopic studies.—A previous investigation (1) disclosed two puzzling features in the 14 (d) 7 duck variant growing in pigeons which have been here studied in more detail. The first was the frequent presence in the tumor of formations resembling atypical inflammatory granulomas, and the second was the occurrence, in some cases, of a myeloid infiltration close to or distant from the tumor, so conspicuous as to make one suspect the existence of a leukotic process.

The granuloma-like lesions were especially present, in the primary tumors or their metastases, around areas showing regressive phenomena (Figs. 1 and 2). These areas were made up of necrobiotic or necrotic tissue surrounded by giant cells. The latter, apparently derived from the tumor cells, had in general an irregular shape and contained hyperchromatic nuclei, resembling foreign body giant cells. In a few cases recent hemorrhage or a slight granulocytic infiltration were present around the layer of giant cells. No foreign bodies, acid-fast bacilli, or any other micro-organisms could be detected on the histological sections. These lesions apparently were determined by two mechanisms:

TABLE 2

EFFECTS OF CELLS AND FILTRATES FROM PIGEON TUMORS ON PIGEONS, DUCKLINGS, AND CHICKS

	Donor host		Recipient hosts					
	Age of Pigeon	Age of Tumor	Pigeons		Ducklings		Chicks	
Exp.	(Mos.)	(Wks.)	C.S.	F.	C.S.	F.	C.S.	F.
1	2	3*	0/5		2/2			
2	2	3*	0/4		2/2			
3†	15	4	0/4	0/6		0/2	3/3	0/5
4	10	5 1/2*	0/4		2/2			
5	2	5 1/2	0/4					
6	2	9	0/9					
7	15	10	0/4					0/5
8‡	5	10	0/1					
9	20	10	0/1	0/4	0/2	0/8	0/2	0/8
10	15	10	0/4					0/5

Figures expressing the results indicate the number of animals developing tumors over the total of animals inoculated.

* Tumor secured by biopsy.

† Pool of two tumors.

‡ Same tumor as that of experiment 2, secured after death.

(*a*) a regressive process, necrobiosis, followed by the giant cell reaction and (*b*) the same reaction, preceded by focal hemorrhage with destruction of the blood cells. The latter mechanism was more frequently observed and seems to afford the most probable explanation for the granuloma-like lesions present in recent metastatic foci. The explanation largely coincides with that given by Rous (9), who also mentioned small giant cells

located especially around regions of degeneration in the Rous sarcoma growing in chickens (10).

Lesions somewhat comparable to the ones found in the tumors were also observed at the site of injection, in the breast muscles of three pigeons, when attempting a second cell passage of pigeon-grown tumors (Fig. 3). In these cases a granulomatous structure was much more evident, the layer of giant cells being surrounded by fibroblasts intermixed with round cells, a few granulocytes, and occasional red blood cells. No foreign body or micro-organism was found. Apparently, these lesions were the consequence of a nonspecific local tissue reaction against the tumor material inoculated and should be considered as a foreign body type of granuloma, in the same class as those produced by Levine (8) in the muscles of chicks injected with suspensions of powdered normal muscle of chickens.[1]

To determine the meaning of the myeloid infiltration around the tumors, a systematic morphological study of the blood was carried out on 26 pigeons which had received successful or unsuccessful injections in the breast muscle of cell suspensions or filtrates from duck or pigeon tumors. Another six normal pigeons were similarly studied. Blood smears were taken at least once before the pigeons were injected and, in most cases, several times afterward, generally at 1–2-week intervals; a final blood examination was always made before the pigeons were killed. In no case were changes in the blood observed that made one suspect that a leukemic condition existed.

In an additional experiment, 1 cc. of either heparinized blood or a suspension of bone marrow cells of tumor-bearing pigeons was injected into the breast muscles of eight pigeons; for a control, 1 cc. of a similar suspension of cells from a normal duckling was injected into three pigeons. No alterations worth recording were observed in any of the inoculated animals.

DISCUSSION

Since filtrates of the duck variant 14 (d) 7 of the Rous sarcoma were effective in adult pigeons and since it was known (4) that filtrates of the same tumor were also effective in adult chickens, a situation was created in which the same virus was capable of inducing sarcomas in mature individuals from three different animal species.

This indicates that, in the process of variation of the original Rous virus, which resulted in its adaptation to ducks, the virus not only kept its pathogenic power for the original species, the chicken, but acquired at the same time a pathogenic power for still another species, the pigeon. Whether the young age of the donor ducks was responsible for the inability of the virus to vary, as is the case in the chicken-duck sequence (3), or whether the virus was inherently incapable of further variation remains to be determined.

The tumor virus became masked in the pigeon tumors, as shown by the ineffectiveness of their filtrates in pigeons, ducklings, and chicks, and became unmasked upon growth of the tumors in ducklings and chicks, as shown by effectiveness of tumor filtrates in other ducklings and chicks. Another manifestation of the masking-unmasking sequence was the absence, in tumor-bearing pigeons, of hemorrhagic lesions—the lesions expressing the effect of a free virus (6)—and the frequent presence of such lesions in the ducklings and chicks in which the pigeon tumor was transplanted. These phenomena are essentially the same as those observed in the original Rous virus which becomes masked in tumors in old chickens and is unmasked in chick tumors (6).[2]

With the Rous sarcoma, virus masking was significantly associated with signs of depression of the growths such as low metastasizing power and low transmissibility by cells, events frequently followed by tumor regression (6). The same phenomena have been observed in the pigeon tumors, for metastases were observed in only three out of ten tumors that grew progressively, and regression was frequent in the first generation, in eleven out of 21 tumors, and was constant, in the second generation, in the birds in which initial growth was observed.[3] Also, the frequent presence of granuloma-

[1] The following observations made on separate pigeons, not included in the tables, are worth mentioning. Three out of nine pigeons, injected intravenously with 1 cc. of filtrate of one of the pigeon tumors, developed, in the liver and lung, lesions which grossly resembled tumors. Microscopically, however, the lesions were typical of tuberculosis, and appropriate stains revealed the presence of acid-fast bacilli. A curious situation was found in one of the pigeons bearing a large tumor in the breast induced by cells from the 14 (d) 7 sarcoma. In this bird, in addition to atypical metastasis in the ribs, were nodules in the lungs and liver (Fig. 4) which could be interpreted as tuberculous granulomas or tumor metastases showing the granuloma-like lesions above described; acid-fast bacilli were not present. Whether the pigeons showing the tuberculous lesions were affected by the disease before inoculation of the tumor material or whether the latter revived a latent tuberculous infection cannot be decided. The observations illustrate possible causes of error when working on pigeon tumors.

[2] This observation, as well as others pertaining to the behavior of the Rous sarcoma in old chickens, is part of an investigation carried out by Dr. P. M. Freire and ourselves, the results of which will soon be published. The main points of this investigation have been summarized in a review (6).

[3] It may be significant in this respect that the only pigeon tumor that failed to grow in ducklings and chicks, in experi-

like formations and the infiltration associated with the tumors were probably indicative of failing tumor growth.

SUMMARY

Filtrates from a variant of the Rous sarcoma, effective in both chickens and ducks, induced tumors in nine out of seventeen pigeons inoculated. Cell suspensions from the same variant induced tumors in all the twelve pigeons inoculated.

From the total of 21 tumors produced by either cells or filtrates, metastases were observed in only three cases, while regression occurred in eleven cases. The tumors could not be maintained by further cell passages in pigeons.

In all the ten pigeon tumors studied in this respect, the virus was found to be masked, as shown by the ineffectiveness of its filtrates in pigeons, chicks, and ducklings. The virus became unmasked in chicks and ducklings, as shown by the effectiveness of filtrates from the tumors induced in these hosts by cell suspensions of the pigeon tumors.

ment 9 of Table 2, was, of all the tumors tested, the oldest tumor growing in the oldest host.

REFERENCES

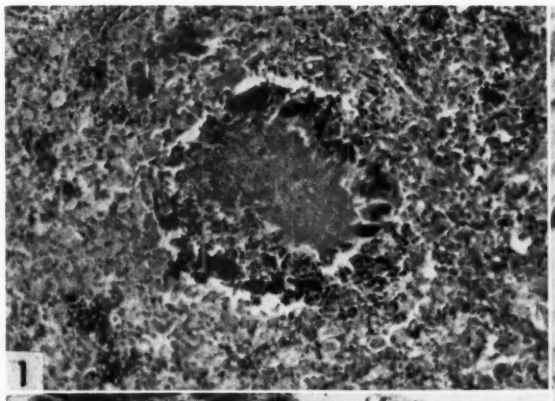
1. Duran-Reynals, F. The Reciprocal Infection of Ducks and Chickens with Tumor-inducing Viruses. Cancer Research, **2**:343–69, 1942.
2. ———. The Infection of Turkeys and Guinea Fowls by the Rous Sarcoma Virus and the Accompanying Variations of the Virus. *Ibid.*, **3**:569–77, 1943.
3. ———. The Age Factor in Adaptability of Sarcoma Virus to Other Animal Species. Science, **103**:748–49, 1946.
4. ———. A Study of Three New Duck Variants of the Rous Chicken Sarcoma. Cancer Research, **7**:99–102, 1947.
5. ———. Transmission to Adult Pigeons of Several Variants of the Rous Sarcoma of Chickens. *Ibid.*, **7**:103–6, 1947.
6. ———. Neoplastic Infection and Cancer. Am. J. Med., **8**:490–511, 1950.
7. ———. The Significance of the Lesions Induced in the Central Nervous System of Ducklings by a Variant of the Rous Sarcoma. Yale J. Biol. & Med., 1950.
8. Levine, M. The Cytology of the Tumor Cell in the Rous Chicken Sarcoma. Am. J. Cancer, **36**:581–602, 1939.
9. Rous, P. A Transmissible Avian Neoplasm. (Sarcoma of the Common Fowl.) J. Exper. Med., **12**:696–705, 1910.
10. ———. A Sarcoma of the Fowl Transmissible by an Agent Separable from the Tumor Cells. *Ibid.*, **13**:397–411, 1911.

Fig. 1.—Breast tumor in pigeon obtained by cell suspension of variant 14 (d) 7 of the Rous chicken sarcoma. Necrotic area surrounded by giant cells. Recent hemorrhage on the lower left-hand side (*). Hematoxylin-eosin. ×120.

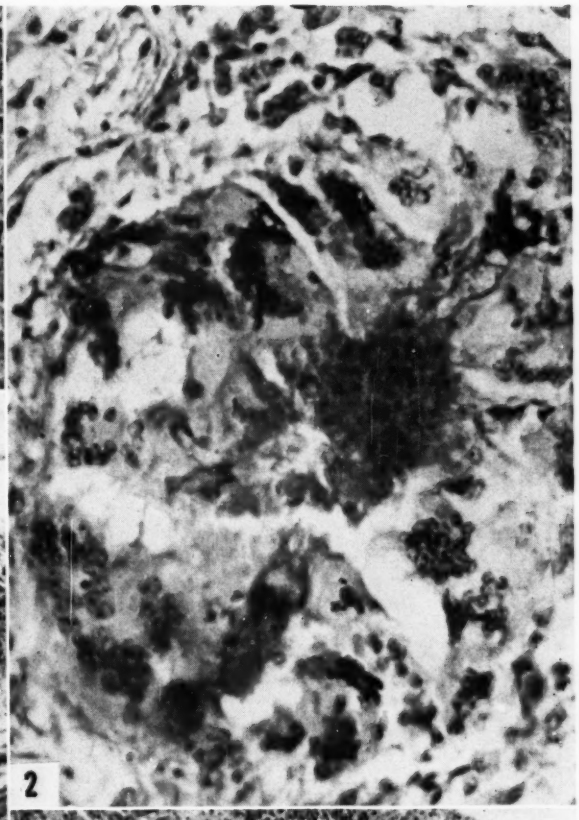
Fig. 2.—Lung. Granuloma-like lesion in a metastasis from a tumor induced in the breast muscle of a pigeon by cell suspension of variant 14 (d) 7 of the Rous sarcoma. Hematoxylin-eosin. ×450.

Fig. 3.—Granuloma developed in the breast muscle of a pigeon injected with cell suspension of pigeon-grown duck tumor 14 (d) 7. Hematoxylin-eosin. ×120.

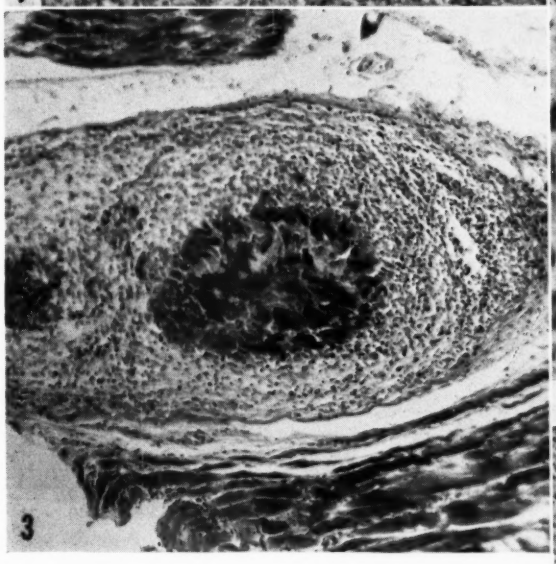
Fig. 4.—Liver of pigeon successfully injected in the breast muscles with cell suspension of duck variant 14 (d) 7 of the Rous sarcoma, showing probable metastases with an area of necrosis surrounded by giant cells. Hematoxylin-eosin. ×120.



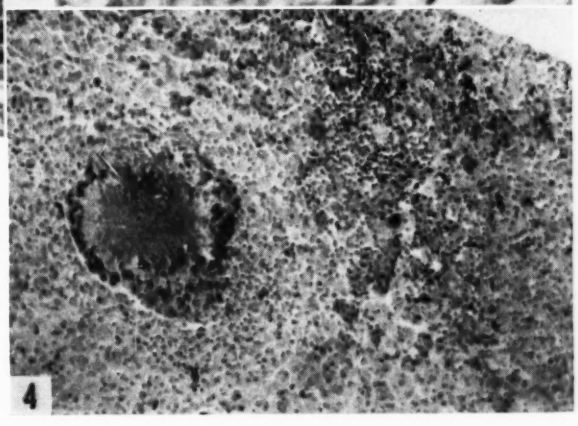
1



2



3



4

The Effect of Pituitary Growth Hormone (Somatotrophin) on the Body Weight and Tumor Growth in C3H Mice Bearing a Transplantable Mammary Adenocarcinoma*

MAURICE C. SMITH, PAUL A. SLATTERY, MICHAEL B. SHIMKIN, CHOH HAO LI, RAYMOND LEE, JOHN C. CLARKE, AND WILLIAM R. LYONS

(Division of Anatomy and Department of Biochemistry, University of California, Berkeley, Calif.; and the Laboratory of Experimental Oncology, National Cancer Institute, National Institutes of Health, Public Health Service, Federal Security Agency, and the University of California Medical Center, San Francisco, Calif.)

The chronic administration of pituitary growth hormone has previously been shown to increase the incidence of neoplastic growth in pulmonary and lymphatic tissues (3), in adrenal glands (4), and in the reproductive organs (5). This paper deals with the effect of somatotrophin (pituitary growth hormone) (2) on the rate of growth of a transplantable mammary adenocarcinoma in C3H mice.

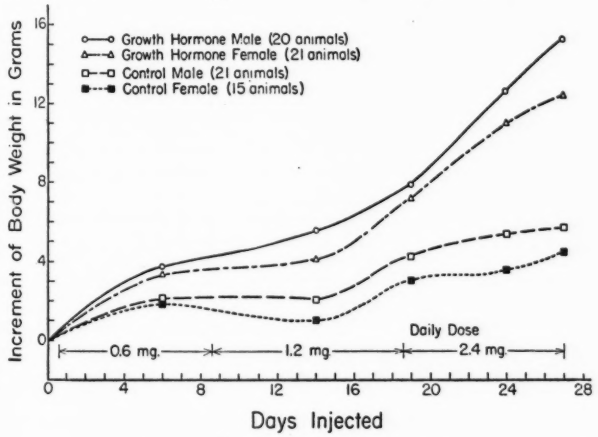


CHART 1.—Showing the effect of growth hormone on the body weights of C3H mice bearing a transplantable mammary adenocarcinoma.

The data presented in this paper represent the combination of two series of experiments repeated under similar conditions. Each series was composed of approximately 40 mice distributed in four groups: (*a*) males receiving somatotrophin; (*b*) females receiving somatotrophin; (*c*) males receiving saline; and (*d*) females receiving saline.

The mice were shaved on the dorsum, and uniform fragments of tumor were implanted by trocar into the dorsal subcutaneous tissues. The donor in both series was a C3H mouse bearing an emulsion-injected mammary adenocarcinoma. The day of implantation was designated as D-day. The mice were weighed on D + 1, D + 6, D + 14, D + 19, D + 24, and D + 27. Administration of somatotrophin[1] was begun on D + 1. Three subcutaneous injections were given daily, with the exception of Saturdays and Sundays in which case two injections were given daily. The first dose of hormone was 0.6 mg/day/mouse. This level was increased periodically (as shown by Chart 1) until the level of 2.4 mg/day/mouse was reached. The mice were sacrificed on D + 27, and the tumors were excised and weighed.

TABLE 1

THE EFFECT OF PITUITARY SOMATOTROPHIN ON THE MEAN TUMOR WEIGHTS IN GRAMS OF C3H MICE BEARING A TRANSPLANTABLE MAMMARY ADENOCARCINOMA

Group	No. of mice	Tumor weight
Control male	19	3.06±0.51*
Control female	15	2.46±0.49
Experimental male	18	5.75±1.07 (<0.05)†
Experimental female	19	4.99±0.90 (<0.02)

* Standard error of the mean.

† The values in the parentheses are the P values of Fisher obtained when the experimental groups are compared with their respective controls.

RESULTS

Control mice injected with saline.—Since this experiment deals with the tumor growth rate, all animals in which the transplant did not grow were discarded from the data. In one out of 21 control males, and in 2 out of 19 control females, the transplant failed to grow. The average tumor weight was 3.06 gm. ± 0.51 gm. in the control male group

* Aided by grants in part from the U.S. Public Health Service and the Lasker Foundation, New York.

Received for publication September 29, 1951.

[1] Female rats hypophysectomized at 27 days of age and 14 days later injected daily for 10 days with a dose of 0.01 mg. of this hormone preparation showed an average body weight increase of 10 gm.

and 2.46 gm. ± 0.49 gm. in the control female group (Table 1).

In the course of the 27-day experiment the male controls gained 5.8 gm., while the female controls gained 4.6 gm. (see Table 2). The regrowth of hair from the shaved areas occurred at a slow rate in the controls of both sexes. Most of the controls presented the usual cachectic appearance near the termination of the experiment. Two of the females and one male in the control groups died on D + 26.

Experimental mice injected with somatotrophin.—Tumor transplants failed to grow in three of the females and two of the males injected with somatotrophin.

The average tumor weight was 4.99 gm. ± 0.90 gm. for the females and 5.75 gm. ± 1.07 gm. for the males (see Table 1). The increase of tumor weight in the hormone-treated mice is quite striking when compared to that of their respective controls (Chart 2). The increase in weight of the tumors was significant at the 5 per cent level for the males and at the 2 per cent level for the females.

The effect of somatotrophin on the body weight of the mice was marked. The males gained 15.3 gm., and the females gained 12.5 gm. When one subtracts the gain of weight of the respective controls from that of the experimental groups, it is seen that somatotrophin was responsible for a 9.5-gm. increase in the males and a 7.9-gm. increase in the females (Table 2 and Chart 1). Hence, it is obvious from these results that somatotrophin induced a body weight increase aside from and in spite of the rapid tumor weight increase. No significant positive correlation between the tumor weights and corrected body weights (Chart 3) could be demonstrated.

Growth of hair occurred at a much faster rate in the hormone-injected animals than in the controls, and after 27 days the experimental groups had nearly complete hair regrowth over the shaved areas, whereas the controls were still nearly barren

TABLE 2

THE EFFECT OF PITUITARY SOMATOTROPHIN ON THE BODY WEIGHT IN GRAMS OF C3H MICE BEARING A TRANSPLANTABLE MAMMARY ADENOCARCINOMA

Group	No. of mice	Weight on D+1 (gm.)	Weight on D+27 (gm.)	Difference between D+1 and D+27 (gm.)	Difference between D+1 and D+27 minus the tumor weight (gm.)
Control male	21	22.6±0.73*	28.4±0.88	+ 5.8	+ 3.2
Control female	15	22.1±0.75	26.7±0.82	+ 4.6	+ 2.0
Experimental male	20	23.2±0.92	38.5±1.30	+15.3 (<0.001)†	+10.0 (<0.001)
Experimental female	21	23.7±0.91	36.2±1.23	+12.5 (<0.001)	+ 7.2 (<0.01)

* Standard error of the mean.

† The values in parentheses are P values of Fisher obtained when the experimental groups are compared with their respective controls.

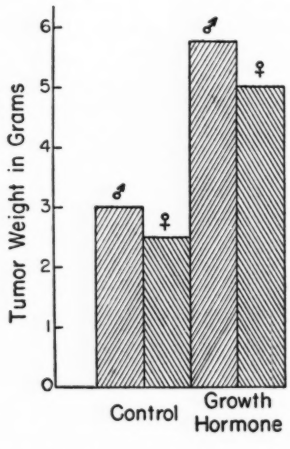


CHART 2.—Showing the effect of growth hormone on the growth of a mammary adenocarcinoma in C3H mice.

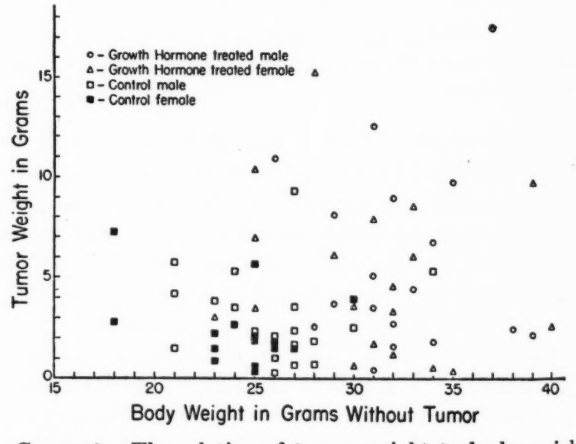


CHART 3.—The relation of tumor weight to body weight without the tumor in C3H mice bearing a transplantable mammary adenocarcinoma, injected with pituitary growth hormone.

in the areas which had been previously shaved.

Of great interest was the observation that the experimental group with the large tumors appeared in better health than the controls; and in none of them were signs of severe cachexia observed. One male in the hormone-treated groups with a tumor weighing 17.28 gm. appeared to be in good health on D + 27. The better appearance of the mice in the somatotrophin groups was unquestionably due in part to the better condition of their pelage.

DISCUSSION

Bischoff and Maxwell (1) reported an increased rate of growth of Carcinoma 256 in rats and a small increased rate of growth of Sarcoma 180 in mice following administration of crude growth-promoting extracts of the pituitary. Sugiura and Benedict (7) also reported that extracts of the anterior pituitary had a stimulating effect on the growth of neoplasms in mice. However, Schulman and Greenberg (6), working with transplanted mammary adenocarcinoma in strain A mice, found that a commercial growth hormone preparation had no appreciable effect on tumor growth. It may have been, however, that in the latter case the hormone was not effective throughout the experiment, since all animals suffered a weight loss after the first week of therapy.

TABLE 3

THE EFFECT OF SOMATOTROPHIN ON THE RELATIVE GROWTH OF A MAMMARY ADENOCARCINOMA IN C3H MICE

Group	Tumor weight as a per cent of the total body weight	Tumor weight as a per cent of the body weight minus tumor weight
Control male	10.4±1.55*	12.7±2.3
Control female	9.3±2.02	11.0±2.7
Experimental male	13.9±1.94 (C.R.=1.21)†	17.5±3.2 (C.R.=1.5)
Experimental female	13.1±2.10 (C.R.=1.31)	16.2±3.0 (C.R.=1.3)

* Standard error of the mean.

† C.R. = critical ratio obtained when the experimental groups are compared with their respective controls.

In this experiment the weight increase of the somatotrophin-injected group over the increase of the controls was significant at the 1 per cent level in both sexes (Table 2 and Chart 1), and, hence, the hormone was effective. The increased tumor weight when compared to that of the respective controls was significant at the 5 per cent level for the males and at the 2 per cent level for the females.

When the tumor weights of the various groups are expressed as a percentage of the body weights (with or without the tumor), and the means of these percentages compared, no statistically significant increase in the tumor weights of the experimental groups was observed (Table 3). However, this might be expected in view of the fact that the hormone increases both the body and tumor weight.

CONCLUSIONS

1. The administration of pituitary growth hormone (somatotrophin) to C3H mice bearing a transplantable mammary adenocarcinoma resulted in a significant increase in body weight of both males and females as compared to the increase of body weight in the controls.

2. In both sexes the tumor weight at the end of 27 days of administration of pituitary somatotrophin was significantly greater than that in the control mice.

REFERENCES

1. BISCHOFF, F.; MAXWELL, L. C.; and ULLMAN, H. J. Hormones in Cancer. VIII. Influence of the Hypophysis. Am. J. Cancer, **21**:329–45, 1934.
2. LI, C. H.; EVANS, H. M.; and SIMPSON, M. E. Isolation and Properties of the Anterior Hypophyseal Growth Hormone. J. Biol. Chem., **159**: 353–66, 1945.
3. MOON, H. D.; SIMPSON, M. E.; LI, C. H.; and EVANS, H. M. Neoplasms in Rats Treated with Pituitary Growth Hormone. I. Pulmonary and Lymphatic Tissues. Cancer Research, **10**:297–308, 1950.
4. ———. Neoplasms in Rats Treated with Pituitary Growth Hormone. II. Adrenal Glands. *Ibid.*, pp. 364–70.
5. ———. Neoplasms in Rats Treated with Pituitary Growth Hormone. III. Reproductive Organs. *Ibid.*, pp. 549–56.
6. SCHULMAN, M. P., and GREENBERG, D. M. Effect of Purified Growth Hormone on Tumor Growth. Proc. Soc. Exper. Biol. & Med., **72**:676–77, 1949.
7. SUGIURA, K., and BENEDICT, S. R. The Influence of Hormones on the Growth of Carcinoma, Sarcoma, and Melanoma in Animals. Am. J. Cancer, **18**:583–602, 1933.

Relation of Uptake of Radiophosphorus into Nucleic Acids to Age of Developing Chick Embryos*

KINGSLEY M. STEVENS†

(Department of Internal Medicine of the Bowman Gray School of Medicine, Wake Forest College, Winston-Salem, North Carolina)

While much work has been done on the turnover rates of nucleic acids in young and adult animals, especially the rat (5), little is to be found on their turnover in embryonic tissue. Since the relative rate of growth of tissue is much greater during embryonic development than during adult life, it was felt that a study of the changes in turnover rates of desoxyribonucleic acid (DNA) and pentosenucleic acid (PNA) in relation to age would be of interest. When mature animals are used, separate organ analyses can be made, but such analyses are not generally feasible on embryonic material. With the use of radiophosphorus,[1] we have demonstrated high turnover rates with large differences in these rates at different ages.

MATERIALS AND METHODS

Fertile chicken eggs were incubated for from 9 to 16 days at 38° C. In each experiment from four to eleven eggs were used, the larger number being used for the younger embryos. Five-tenths ml. of a solution containing 5 μc. of carrier-free $Na_2HP^{32}O_4$, 25 units of penicillin, and 250 μg. of streptomycin was injected into the yolk sac of each egg. After 6 hours' further incubation at 38°, the embryos were dissected free, observed for viability, pooled, weighed, and blended. The nucleic acids were then extracted by the method of Hammarsten (4), with the modification that the PNA was precipitated by the neutralization of the acid lanthanum after the DNA was removed. Although a great deal of PNA is lost by this technic, the loss does not alter the specific activity. Phosphorus determinations on aliquots of the DNA and PNA were made by the method of Allen (2) and DNA determinations by the method of Stumpf (9). When a value of DNA phosphorus of 9.1 per cent was assumed, the DNA values as determined by the two methods checked within 10 per cent. DNA analyses were made on the PNA to detect possible contamination. Only one experiment showed contamination of over 6 per cent for which appropriate corrections were made. Counts were made on the entire dried sample brought to constant mass with sodium chloride. Over 10,000 counts in duplicate were taken in all cases.

Since the administered $Na_2HP^{32}O_4$ equilibrated with the total inorganic P of the egg, it was necessary to multiply the "raw" specific activity values by the quantity of inorganic P per egg. These data for eggs of varying ages were secured from Kugler (6). The term "relative specific activity" as used here refers to the product of counts/minute/milligram nucleic acid P $\times$ the milligrams of inorganic P per egg. Since the same quantity of P^{32} was injected in all experiments, this term is proportional, though not equal, to the more conventional definition of "relative specific activity."

RESULTS

The results are shown in Chart 1.

The "relative specific activities" of both PNA and DNA are seen to rise rapidly from the ninth day, reach a peak on the thirteenth day, and then drop precipitously. The DNA remains low through the sixteenth day, while the PNA begins to rise moderately. The ratio of PNA/DNA "relative specific activities" remains essentially constant until the fourteenth day, and then rises.

DISCUSSION

Novikoff and Potter (8) studied the changes in PNA and DNA concentrations in the developing chick embryo. Their data are shown in Chart 2, along with data from Needham (7), showing changes in protein concentrations.

In our study only 9–16-day embryos were in-

* Supported, in part, by a contract between the Atomic Energy Commission and the Bowman-Gray School of medicine.

† Atomic Energy Commission postdoctoral fellow in the Medical Sciences, 1948–50. Present address: Surgical Research Unit, Brooke Army Hospital, Fort Sam Houston, Texas.

[1] It has been demonstrated that inorganic P^{32}- and C^{14}-labeled purines give similar nucleic acid turnover rates (1).

Received for publication October 6, 1951.

vestigated. Younger embryos required a larger number of eggs than were available, while older ones could not be extracted satisfactorily by this technic.

The similarity of the position of the peaks in Charts 1 and 2 is apparent. Chart 2 represents concentrations of these substances, while Chart 1

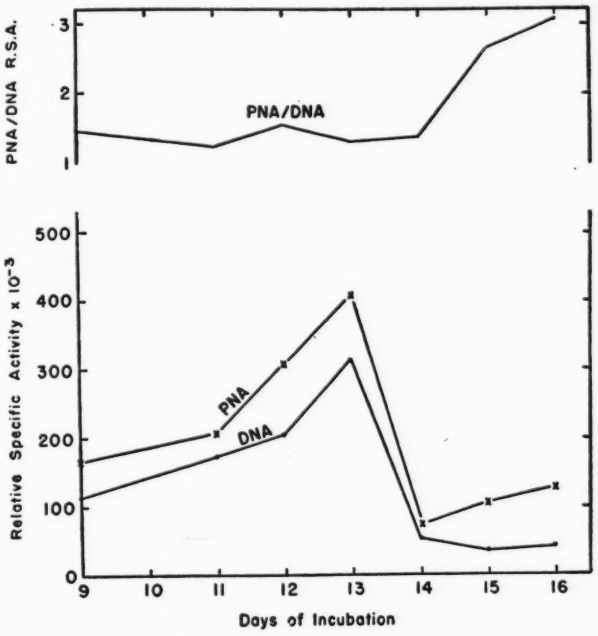


CHART 1.—Changes in PNA and DNA relative specific activities (counts/min/mg nucleic acid P×mg inorganic P/egg) with increasing age. No values were obtained for the tenth day. Days 9 through 13 represent single experiments. Day 14, three experiments, standard deviation PNA, 24,300; DNA, 18,000. Day 15, four experiments, s.d. PNA, 19,600; DNA, 11,200. Day 16, two experiments, s.d. PNA, 1230; DNA, 1520.

presents the pattern of change in turnover rates. Hence, not only do the concentrations but also the turnover rates increase greatly about the thirteenth day. Since it is difficult to determine accurately just how long an egg has been incubating, the difference of 1 day in the peak is probably not significant. If both the turnover rates and the amounts of nucleic acids are important factors in the rate of protein synthesis, then around the thirteenth or fourteenth day the product of the two is at a level several times higher than at earlier or later periods between 9 and 16 days.

A rough estimation of the absolute turnover rates can be calculated.[2] For the peak value of the thirteenth day, a rate of 148 per cent per 24 hours is found for PNA and 116 per cent for DNA. This contrasts strongly with the rate for adult animals, since the highest nucleic acid turnover rates in rabbits, found in bone marrow, were only 13 per cent for PNA and 4 per cent for DNA per day.[3] Even the lowest rates in the embryo, found on the fourteenth day, are 28 per cent and 20 per cent per day for PNA and DNA, respectively.

After the thirteenth day the DNA activity drops and then remains constant, while the PNA activity drops but quickly begins rising. This may reflect a continued high rate of cytoplasmic protein synthesis in contrast to a relative decrease in cell division.

Since these studies were made on whole embryos, the particular organs responsible for the increase remain unknown. Davidson and Leslie (3) could demonstrate no marked increase in the amounts of nucleic acids in the heart or liver of chick embryos in the twelfth- to sixteenth-day period. However, in adult animals, the nucleic acid turnover rates are much higher in lymphoid tissue, spleen, bone marrow, and intestinal mucosa (5).[3] Study of these tissues in embryos would be preferable if sufficient quantities of tissues could be secured.

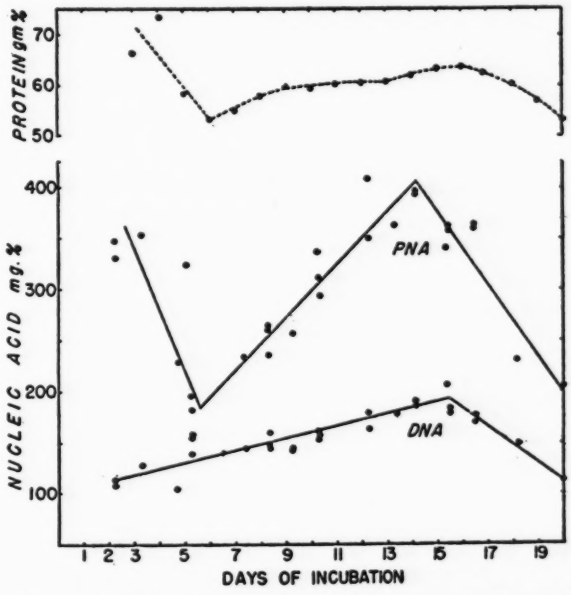


CHART 2.—Changes in PNA and DNA concentrations with age, from Novikoff and Potter (8). Top curve: changes in protein concentration, from data of Needham (7). Dry weight basis.

It is clear that, about the thirteenth or fourteenth day, the chick embryo undergoes drastic changes in its nucleic acid metabolism. Further study of this period may reveal important information concerning normal growth.

[2] Method of calculation: 5 μc. P^{32} injected which produce 11×10^6 disintegrations per minute. The counter efficiency for P^{32} was 9.9 per cent. Hence, 5 μc. would produce 10.9×10^5 counts per minute. There are 17.2 mg. of inorganic P in a 13-day egg. This will give a specific activity of 6.3×10^4 counts/min/mg P. The PNA-P specific activity was 23.6×10^3 counts/min/mg PNA-P. Hence, the turnover rate would be 37 per cent for 6 hours, or 148 per cent per day.

[3] K. M. Stevens, unpublished data.

SUMMARY

1. The uptake of radiophosphorus into desoxyribonucleic acid (DNA) and pentosenucleic acid (PNA) in 9–16-day chick embryos has been determined.

2. The "relative specific activities" of both DNA and PNA rise rapidly to a peak on the thirteenth day, then fall abruptly to about 20 per cent of this peak value. The DNA activity then continues constant, while the PNA begins to rise again.

3. The turnover rates of both nucleic acids, but especially DNA, appear to be much higher in embryos than in adult tissues.

REFERENCES

1. Abrams, R., and Goldinger, J. M. Utilization of Purines for Nucleic Acid Synthesis in Bone Marrow Slices. Arch. Biochem., **30**:261–68, 1951.
2. Allen, R. J. L. The Estimation of Phosphorus. Biochem. J., **34**:858–65, 1940.
3. Davidson, J. N., and Leslie, I. Nucleic Acids in the Development of the Chick Embryo: Changes in Ribonucleic Acid Phosphorus (RNAP) and Desoxyribonucleic Acid Phosphorus (DNAP) in Heart and Liver. Biochem. J., **43**: 266, 1948.
4. Hammarsten, E. On the Extraction of Nucleotides from Cells. Acta Med. Scandinav. (Suppl.), **196**:634–45, 1947.
5. Hevesy, G. Advances in Enzymology, **7**:173–82. New York: Interscience, 1947.
6. Kugler, O. E. Acid Soluble Phosphorus in the Developing Hen's Egg. J. Cell. & Comp. Physiol., **23**:69–75, 1944.
7. Needham, J. Chemical Embryology. New York and Cambridge: Macmillan, 1931.
8. Novikoff, A. B., and Potter, V. R. Changes in Nucleic Acid Concentration during the Development of the Chick Embryo. J. Biol. Chem., **173**:233–38, 1948.
9. Stumpf, P. K. A Colorimetric Method for the Determination of Desoxyribosenucleic Acid. J. Biol. Chem., **169**:367–71, 1947.

Effect of Neoplastic Tissue on the Turnover of Liver Nucleic Acids*

ANITA H. PAYNE, LOLA S. KELLY, AND MARGARET R. WHITE

(Donner Laboratory of Medical Physics, University of California, Berkeley, Calif.)

In two earlier papers (5, 7), the authors reported an increased rate of incorporation of P^{32} into the desoxyribonucleic acid (DNA) of livers and spleens in mice and rats bearing transplanted tumors and in pregnant mice and rats. The present paper deals with an extension of this work to some other tumors and with measurements of the rate of P^{32} incorporation into the nuclear and cytoplasmic pentosenucleic acid (PNA) of these tissues.

According to current theories, PNA is intimately connected with protein synthesis and so might show a higher turnover in animals undergoing rapid tissue growth. If this were the case and if PNA and DNA had a common precursor, then DNA might show a higher specific activity not due to a change in its turnover but due only to the increased specific activity of its precursor. The present experiment was an attempt to test this possibility.

METHODS

All mice were fasted for 24 hours prior to the experiment. They received intraperitoneal injections of P^{32}-labeled sodium phosphate in isotonic saline at pH 7 about 9 A.M. and were killed with ether exactly 4 hours later. The livers were removed, and the isolation of the nucleic acids was begun as soon as possible. In order to obtain enough material, the livers from eight or more mice were pooled.

Each animal received approximately 30 μc. of P^{32}-labeled sodium phosphate. The procedure used for the nucleic acid isolations was an adaptation of several published methods and will be given in detail. In view of previous experience with active contaminants, it was felt that it was important to have the final nucleic acids in such a form that they could be easily reprecipitated to constant specific activity.

The method of Barnum *et al.* (2) was followed for the separation of the nuclei from the cytoplasm and the subsequent purification of the nuclei and separation of nuclear pentosenucleoprotein (PNA-protein) from desoxypentosenucleoprotein (DNA-protein).

After being cooled the livers were forced through a tissue press with holes about 1 mm. in diameter and were homogenized 2–3 minutes in 4 volumes of ice-cold saline (0.85 per cent NaCl containing 2 ml. 0.1 N NaOH per liter). Homogenization was carried out in a Potter-Elvehjem tube. The homogenate was centrifuged 4 minutes at 1,400 $\times$ *g* in a No. 1 International centrifuge operated in a cold room. The resultant supernatant fluid was siphoned off and stored in the refrigerator until the following day for the isolation of the cytoplasmic PNA. The nuclear sediment was suspended in 2 per cent cold citric acid and centrifuged for 10 minutes in a clinical centrifuge at 500 $\times$ *g*. The sediment was resuspended and centrifuged 14 times at the same speed, after which treatment the supernatant fluid was perfectly clear. The centrifugations were carried out as follows: once for 10 min., twice for 5 min., 5 times for 3 min., and 6 times for 1 min. The isolated nuclei were washed twice with saline to remove citric acid and then were extracted in 95 per cent methyl alcohol, followed by three extractions with boiling alcohol-ether (3:1), and a final alcohol extraction. The lipid-free nuclei were then suspended in an ice-cold buffer at pH 10 prepared by mixing equal volumes of 0.1 M Na_2CO_3 and 0.1 M $NaHCO_3$. The suspensions were kept in an ice-water bath for 30 minutes and then were centrifuged in a clinical centrifuge for 3 minutes. The sediments containing the DNA-protein were washed twice with the cold buffer solution. The supernatant and washes containing the PNA-protein were combined and made 5 per cent to trichloroacetic acid (TCA) and centrifuged in a No. 1 International centrifuge.

The precipitate which represents the nuclear PNA-protein and any possible residual DNA-protein was washed once in 5 per cent TCA. In order to obtain the nucleic acid in a reprecipitable form,

* Partially supported by the United States Atomic Energy Commission.

Received for publication October 6, 1951.

the method from this point on differs from the method of Barnum *et al.* To remove the residual DNA-protein from the PNA-protein, the nucleoprotein precipitate was suspended in 0.14 M NaCl and brought to pH 7. DNA-protein is insoluble in this concentration of NaCl and could be removed by centrifugation. The PNA-protein contained in the supernatant fluid was precipitated out by bringing the pH of the solution down to ~3 with glacial acetic acid and separated by centrifugation. The PNA-protein was then suspended in ice-cold 5 per cent NaOH and kept at 0° ± 2° for 2 hours (4). This split the protein from the nucleic acid. Essentially no decomposition of nucleic acid occurs at this temperature. The protein was precipitated out by adding glacial acetic acid until the solution was just acid to litmus (pH was ~6.5). The solution was centrifuged and the supernatant fluid filtered through tissue paper. The PNA remained in the supernatant fluid and was precipitated by bringing the solution to pH 2 by the addition of HCl and then adding an equal volume of 95 per cent methyl alcohol and centrifuging. This precipitate was dissolved in 0.05 M $NaHCO_3$. Several reprecipitations could be carried out at this point until the nucleic acid maintained a constant specific activity. It was found that the specific activity did not change significantly after reprecipitation.

The DNA was obtained from the gelatinous sediment that remained after the pH 10 extraction by following a modified Beck and Klein method previously published (6).

The cytoplasmic PNA was precipitated from the cytoplasmic extract which had been obtained earlier. TCA was added to a concentration of 5 per cent, the extract was centrifuged, and the supernatant fluid containing the acid soluble phosphorus was decanted. The sediment was washed once with 5 per cent TCA and then suspended in cold 5 per cent NaOH. The procedure from here on was the same as for the nuclear PNA.

As is shown in Table 1, with the use of either the orcinol or the diphenylamine reagent, the density of the color developed per microgram of phosphorus in the nucleic acids isolated by the above method agreed well with that of the commercial preparations. The diphenylamine reagent and the orcinol reagent were prepared according to the directions given by Barnum *et al.* (2). The density was determined with a Beckman DU spectrophotometer. There was essentially no contamination of cytoplasmic PNA or nuclear PNA with DNA as determined by the Feulgen reaction and by the diphenylamine reagent.

The specific activity of the purified nucleic acid was determined in the following manner. One aliquot of the sodium nucleate solution was used for the determination of the phosphate concentration by the method of Fiske and SubbaRow (3).

TABLE 1

ANALYTICAL COMPARISON OF DIFFERENT PREPARATIONS OF NUCLEIC ACIDS

	Cytoplasmic PNA	Nuclear PNA	Commercial* PNA	DNA	Commercial* DNA
E (diphenylamine reaction)†				0.018 ±0.007	0.0194 ±0.006
E (orcinol reaction)‡	0.123 ±0.018	0.113 ±0.013	0.111 ±0.011		
N:P	1.68	1.80	1.88	1.90	1.84§
Feulgen test	0	0	0	+	+

* This material was obtained from the Nutritional Biochemical Corporation, Cleveland, Ohio.

† Optical density at 600 mμ wave length, calculated per μg, P in sample.

‡ Optical density at 660 mμ wave length, calculated per μg. P in sample.

§ The Nutritional Biochemical Corporation's N:P ratio is given as 1.71.

A second aliquot was used for counting the P^{32} with a Victoreen geiger tube.

RESULTS AND DISCUSSION

The results of our measurements are given in Tables 2–4. Each value represents the specific activity of the nucleic acid obtained from the pooled livers of at least eight animals. In the cytoplasmic PNA determinations enough material was available so that each sample was divided into two at the beginning of the isolation procedures, and the two separate determinations were averaged to give the values in the table. The duplicates agreed to better than 10 per cent.

As can be seen from the tables, the increased specific activity of liver DNA, observed earlier in female A strain mice, occurs also in male A strain mice bearing the transplanted mammary carcinoma, in female A strain mice bearing transplanted sarcoma A274, and in female C57 strain mice bearing a transplanted mammary carcinoma.

Contrary to our expectations, there was no significant difference in the rate of incorporation of P^{32} in the cytoplasmic PNA of the livers of tumor-bearing mice. However, the individual variations are rather large, so that a small effect would not necessarily be noticed. Furthermore, our cytoplasmic PNA is obtained from a mixture of the

various cytoplasmic fractions, and there might be a considerable change in one of these fractions without its being evident in our values.

The nuclear PNA specific activities of the tumor group show a statistically significant decrease from those of the control group (Table 2). However, it is felt that these measurements are to be viewed with caution. The ratio between the nuclear and cytoplasmic PNA specific activities is much lower than that obtained by Barnum and Huseby (1), indicating that our nuclear PNA may still be contaminated, possibly with cytoplasmic PNA.

It is clear from these experiments, however, that there is no drastic change in the PNA metabolism of the livers of tumor-bearing hosts. The in-

TABLE 2

SPECIFIC ACTIVITIES × 10^4 OF LIVER NUCLEIC ACIDS IN ♂ A STRAIN MICE BEARING MAMMARY CARCINOMAS

WEIGHT AND AGE OF TUMORS	DNA*		CYTOPLASMIC PNA		NUCLEAR PNA	
	Tumor	Control	Tumor	Control	Tumor	Control
0.17 gm.	1.72	1.09	17.6	11.2	55.3	53.8
7.5 days	1.96	1.37	16.8	11.9	62.6	63.8
	1.44	0.69	16.0	12.4	53.0	66.8
	1.73	1.06	14.1	10.8		
	2.02		11.9			
	1.68		11.4			
	1.41					
1.0 gm.	3.75	1.25	12.5	7.2	39.7	45.4
10 days	4.91	0.71	11.6	9.3	45.2	49.3
	5.45		11.6		58.2	
2.0 gm.	2.22	0.53	13.6	11.5	43.1	55.3
15 days	1.89	0.75	12.0	12.1	35.7	48.5
	2.24		4.2	13.5	41.9	
	5.08		8.2		36.6	
			11.8		48.5	
2.4 gm.	1.55	0.93	7.3	9.1	37.8	51.5
18 days	1.70		7.4		34.7	
Av.	2.55±0.37	0.97±0.11	11.7±1.0	10.9±0.7	45.6±2.7	55.3±2.9
Probability of significance (P)	*0.002*		0.27		*0.013*	

* The values given represent the number of P^{32} counts per milligram of phosphorus divided by the number of counts injected, normalized for the weight of the mice. Errors are expressed as standard deviations of the mean. Significant differences are italicized.

TABLE 3

SPECIFIC ACTIVITIES × 10^4 OF LIVER NUCLEIC ACIDS IN ♀ C57 MICE BEARING MAMMARY CARCINOMAS

WEIGHT AND AGE OF TUMORS	DNA		CYTOPLASMIC PNA		NUCLEAR PNA	
	Tumor	Control	Tumor	Control	Tumor	Control
2.4 gm.	3.65	0.56	9.2	7.8	60.0	
11 days	4.09	0.63	9.9	7.7	45.0	
	3.21		7.8			
5.2 gm.	13.0	0.27	13.2	10.6	79.0	50.0
14 days	10.05	0.44	9.3	10.2		
Av.	6.8±2.0	0.48±0.08	9.9±1.0	9.1±0.8		
Probability of significance (P)	*0.028*					

TABLE 4

SPECIFIC ACTIVITIES × 10^4 OF LIVER NUCLEIC ACIDS IN ♀ A STRAIN MICE BEARING SARCOMA A274

WEIGHT AND AGE OF TUMORS	DNA		CYTOPLASMIC PNA		NUCLEAR PNA	
	Tumor	Control	Tumor	Control	Tumor	Control
0.55 gm.	2.39	0.75	12.0	10.6	56.5	47.0
7 days	2.63	0.88	12.8	11.4		
	3.00		13.1			
2.0 gm.	2.28	0.55	12.1	12.1		
12 days	2.28	0.76	13.6	14.1		
	2.05		20.9			
Av.	2.44±0.14	0.74±0.09	14.1±1.5	12.1±0.8		
Probability of significance (P)	*0.001*		0.14			

crease in the DNA specific activity is apparently due to a true change in its turnover rate and not to a secondary effect associated with PNA turnover or a change in over-all phosphate metabolism.

In a series of 48 female A strain mice bearing transplants of mammary carcinoma (1.3 gm. per mouse), the average liver weight was found to be 7.57 per cent ± 0.18 per cent of the body weight. In eighteen control animals the average liver weight was 6.19 per cent ± 0.17 per cent of the body weight. This indicates a 20 per cent greater liver weight in tumor-bearing animals. Recently, Reddy and Cerecedo (9) reported an increased content of both DNA and PNA in the livers of mice bearing Crocker sarcoma 180 transplants. Kosterlitz and Campbell (8) found an increase in the DNA content of livers of pregnant rats. These observations suggest that in the livers of tumor-bearing mice the increased DNA turnover is an indication of increased cell proliferation.

SUMMARY

The rate of incorporation of P^{32} into DNA, nuclear PNA, and cytoplasmic PNA of liver tissue was measured in male A strain mice bearing transplanted mammary carcinoma, in female A strain mice bearing transplanted sarcoma A274, and in female C57 strain mice bearing a transplanted mammary carcinoma. An increase of P^{32} incorporation into the liver DNA was noted in all three groups of tumor-bearing mice when compared to the controls. A decrease in P^{32} incorporation into nuclear PNA was observed in the male A strain mice bearing mammary carcinoma. No significant difference in the rate of incorporation of P^{32} into the cytoplasmic PNA was observed in the tumor-bearing mice.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Dr. Hardin B. Jones for his continual interest and guidance during the course of the experiments. We also wish to thank Mrs. Jean Siri for transplanting the tumors.

REFERENCES

1. Barnum, C. P., and Huseby, R. A. The Intracellular Heterogeneity of Pentose Nucleic Acid as Evidenced by the Incorporation of Radiophosphorus. Arch. Biochem., **29**:7–26, 1950.
2. Barnum, C. P.; Nash, C. W.; Jennings, E.; Nygaard, O.; and Vermund, H. The Separation of Pentose and Desoxypentose Nucleic Acids from Isolated Mouse Liver Cell Nuclei. Arch. Biochem., **25**:376–83, 1950.
3. Fiske, C. H., and SubbaRow, Y. The Colorimetric Determination of Phosphorus. J. Biol. Chem., **66**:375–400, 1925.
4. Johnson, T. B., and Harkins, H. H. Researches on Pyrimidines. CVII. The Examination of Yeast Nucleic Acid for 5-Methylcytosine. J. Am. Chem. Soc., **51**:1779–84, 1929.
5. Kelly, L. S., and Jones, H. B. Effect of Neoplastic Tissue on the Turnover of Desoxypentose Nucleic Acid. Science, **111**:333–34, 1950.
6. ———. Effects of Irradiation on Nucleic Acid Formation Proc. Soc. Exper. Biol. & Med., **74**:493–97, 1950.
7. Kelly, L. S.; Payne, A. H.; White, M. R.; and Jones, H. B. The Effect of Neoplasia and Pregnancy on the Tissue Desoxypentosenucleic Acid. Cancer Research, **11**:694–96, 1951.
8. Kosterlitz, H. W., and Campbell, R. M. Composition and Structure of the Liver Cell in Pregnancy. Nature, **160**: 676–77, 1947.
9. Reddy, D. V. N., and Ceredo, L. R. Effect of Transplanted Tumors on Nucleic Acid Content of Mouse Tissues. Fed. Proc., **10**:236, 1951.

Macromolecular Particles Obtained from Human Neoplastic and Non-neoplastic Lymph Nodes

II. Electron Microscope Study*

MIRIAM S. HOSTER, QUENTIN VAN WINKLE, JACOB G. RABATIN, AND HERMAN A. HOSTER†

(Departments of Medicine and Chemistry, The Ohio State University, Columbus 10, Ohio)

INTRODUCTION

The present investigation concerns the electron microscope study and the statistical evaluation of histograms of particles, 10–350 mμ, obtained from Hodgkin's disease, leukemia, lymphosarcoma, reticulum-cell sarcoma, carcinoma, and miscellaneous non-neoplastic lymph node cells. The investigation is a continuation of a comparative study which includes electrophoretic, electron microscopy, optical ultracentrifugation, and chemical studies of the isolated neoplastic and non-neoplastic cellular components.

Histograms have been used by Evans and Melnick (2) in estimating the average size distribution of particles obtained from chicken pox and herpes simplex materials, which were photographed in the electron microscope, and by Porter and Thompson (5) in estimating the size range of the virus-like bodies found in mammary carcinoma cells.

The use of differential centrifugation for the segregation of particulate components from broken cell suspensions of many tissues has been investigated extensively during the past few years. Employing this technic, it has been possible to study the physical and chemical nature of the isolated cellular particles in a relatively pure state. In a recent review of the literature, Schneider and Hogeboom (6) have discussed the results of many of these studies.

One important factor in the segregation of macromolecular particles from cellular homogenates is the prevention of particle agglutination during isolation and purification in a liquid medium. Hogeboom, Schneider, and Pallade (3) have developed a method for preventing the agglutination of cell particles from rat and mouse liver during isolation by employing sucrose (0.25 M and 0.88 M) as the tissue-suspending medium. Hoster *et al.* (4) have employed a ten-step centrifugation procedure for the isolation of dispersed particulate components from human lymph node cells in which the suspending medium consists of an isotonic phosphate buffer containing the anticoagulant, heparin; this procedure was used in the present investigation.

MATERIALS AND METHODS

Human lymph nodes obtained at surgery were frozen rapidly to a temperature of −65° C. in a mixture of alcohol and dry ice; the tissues were kept at this temperature until used. A total of eighteen Hodgkin's disease, eight lymphomas other than Hodgkin's, two carcinomas, and thirteen non-neoplastic lymph node preparations was used in the present study.

The method of tissue suspension and the centrifugation procedure (Method V) published previously (4) were followed, with the exception that the tissue suspensions were prepared by grinding in the Waring Blendor for 30–60 seconds, and a vacuum refrigerated Spinco preparative centrifuge, Model L, with a No. 40 Rotor was used in the centrifugation steps requiring gravities of 6,000 and 25,000. The tissue-suspending medium consisted of 0.1 M phosphate buffer, pH 8.0, containing 0.03 per cent heparin.[1] Tissue grinding, extraction, and centrifugation were conducted in a cold room at 4° C.

Samples to be studied in the electron microscope were prepared routinely as follows: One drop of a suspension of the high gravity (25,000 *g*) sediment was allowed to remain for 5 minutes in

* This investigation was supported by research grants from the National Cancer Institute of the National Institutes of Health, United States Public Health Service, and the Ohio Department of Health.

† Deceased May 14, 1951.

Received for publication October 8, 1951.

[1] The sodium heparin used in this experiment was supplied through the courtesy of the Upjohn Company, Kalamazoo, Mich.

contact with formvar(0.4 per cent)-coated electron microscope screens, and the remaining fluid was removed by suction. After the screens were dried over phosphorus pentoxide for 24 hours, they were left in contact with the vapors of 2 per cent osmic acid for 2 hours. The screens were washed with filtered distilled water several times and redried for at least 24 hours before examination in the electron microscope. Visual examination of samples was made in the R.C.A. type E.M.U. electron microscope, and photographs were taken at magnifications of 5,000 and 10,000.

For the construction of histograms, the electron microscope photographic negatives were projected at a magnification of 50,000× and 100,000×, and the diameters of approximately 1,000 particles, representing approximately 15 electron microscope screen areas per sample, were measured.

EXPERIMENTAL PROCEDURES AND RESULTS

Comparison of histograms.—Electron micrographs of the particles obtained in the high gravity (25,000 *g*) sediments from neoplastic and non-neoplastic lymph nodes are shown in Figures 1 and 2. Histograms (Chart 1) of particles photographed in the electron microscope were constructed by plotting, as the ordinate, the percentage of particles in each size range and, as the abscissa, the size range of particles in millimicrons (10–350 mμ). The most striking characteristic of the four general groups (Hodgkin's disease, other lymphoblastomas, carcinomas, and non-neoplastic diseases) of lymph nodes studied is the percentage of particles smaller than 60 mμ. In the Hodgkin's disease group, in five of seven histograms of particles whose diameters were measured at 100,000×, the maximum concentration of particles was found in the 10–20 mμ size range. It should be noted that, although particles smaller than 10 mμ exist in all samples studied, the diameters of particles smaller than 10 mμ could not be measured accurately at a magnification of 100,000; therefore, these measurements are not included in the histograms. In the samples projected at a magnification of 50,000, the smallest particle which could be measured accurately was 20 mμ. In the group of lymphoblastomas other than Hodgkin's disease, the greatest concentration of particles was also in the 10–20 mμ size range. The maximum concentration of particles in the two cases of carcinoma was in the 20–30 mμ size range; and in the non-neoplastic preparations as a group, there was no predominant size range.

The reproducibility of results, based on the technic described, was determined. In thirteen experiments, two samples of the same lymph node were centrifuged on separate occasions; the particles in the high gravity sediments were measured, and histograms were constructed. Chart 2 contains examples of histograms of duplicate lymph node preparations. In the first instance, approximately 1,000 particles were measured in each preparation; and, in the second instance, approximately 1,000 particles were measured in the first preparation and approximately 200 were measured in the second preparation. It was found from this study that, when a sufficient number of particles is measured, the histograms are reproducible. When, however, an insufficient number is measured, duplicate histograms do not resemble each other.

Statistical evaluation of histograms.—In the case of each histogram, the mean particle size, standard deviation, and percentage of particles from 10–60 mμ were determined. From these data the average of each of these values was computed for each disease group (Table 1). The data obtained from the results at magnifications of 50,000× and 100,000× were computed separately. It was found, when the results of the particles measured at 100,000× were analyzed, that the mean particle size of the lymphoma group other than Hodgkin's disease was smaller (27 mμ) than that of the Hodgkin's disease group (42 mμ). The mean particle sizes of the carcinoma and non-neoplastic groups were identical—59 mμ. When the results for the particles measured at 50,000× were analyzed, it was found that the mean particle size of the Hodgkin's disease group was 45 mμ and that of the non-neoplastic preparations was 77 mμ.

The average standard deviation in Hodgkin's disease, carcinomas, and non-neoplastic preparations were in close agreement, 40–46, while that of the lymphomas other than Hodgkin's disease was 23.

On investigation of the distribution of particle sizes from 10–60 mμ, it was observed that the groupings of the particles in the Hodgkin's disease preparations and in the other lymphomas resembled one another, while the groupings of the carcinoma preparations more closely resembled those of the non-neoplastic series.

Histologic cell types in relation to histograms.—A comparison was made between the form of the histograms and the cytology of the lymph nodes. Differential cell counts including twenty oil immersion fields (950×) at random locations throughout the lymph node sections were made. No correlation could be found between the histologic cell type and the percentage of particles

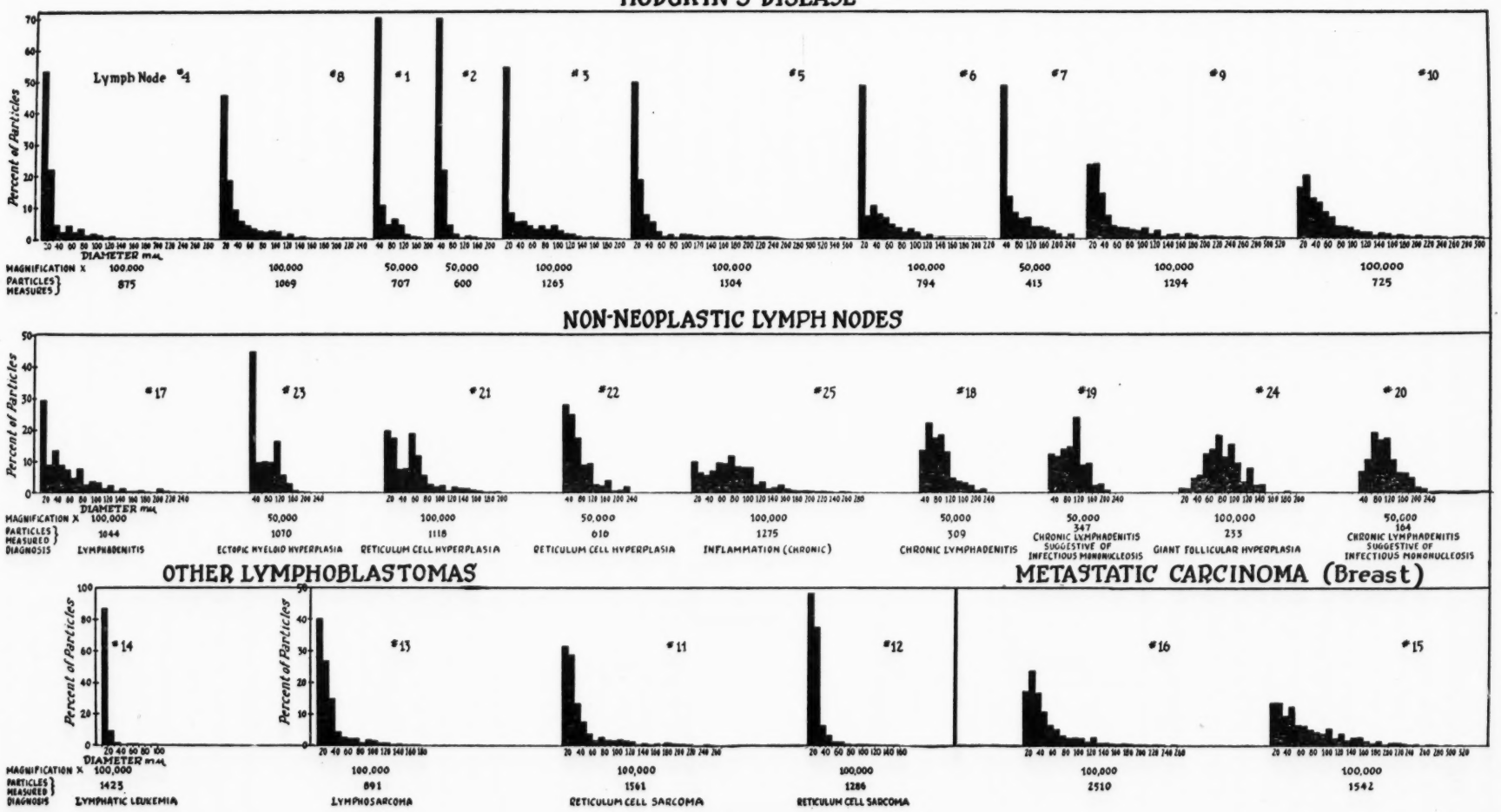

HODGKIN'S DISEASE
Lymph Node #4
#8
#1
#2
#3
#5
#6
#7
#9
#10
Percent of Particles
DIAMETER mμ
MAGNIFICATION X 100,000 100,000 50,000 50,000 100,000 100,000 100,000 50,000 100,000 100,000
PARTICLES MEASURED 875 1069 707 600 1263 1304 794 413 1294 725
NON-NEOPLASTIC LYMPH NODES
#17
#23
#21
#22
#25
#18
#19
#24
#20
MAGNIFICATION X 100,000 50,000 100,000 50,000 100,000 50,000 50,000 100,000 50,000
PARTICLES MEASURED 1044 1070 1118 610 1275 309 347 233 164
DIAGNOSIS LYMPHADENITIS ECTOPIC MYELOID HYPERPLASIA RETICULUM CELL HYPERPLASIA RETICULUM CELL HYPERPLASIA INFLAMMATION (CHRONIC) CHRONIC LYMPHADENITIS CHRONIC LYMPHADENITIS SUGGESTIVE OF INFECTIOUS MONONUCLEOSIS GIANT FOLLICULAR HYPERPLASIA CHRONIC LYMPHADENITIS SUGGESTIVE OF INFECTIOUS MONONUCLEOSIS
OTHER LYMPHOBLASTOMAS
METASTATIC CARCINOMA (Breast)
#14
#13
#11
#12
#16
#15
MAGNIFICATION X 100,000 100,000 100,000 100,000 100,000 100,000
PARTICLES MEASURED 1425 891 1561 1286 2510 1542
DIAGNOSIS LYMPHATIC LEUKEMIA LYMPHOSARCOMA RETICULUM CELL SARCOMA RETICULUM CELL SARCOMA

CHART 1

smaller than 60 mμ or the mean particle size (Table 2), since lymph nodes with similar cytologic pictures yielded submicroscopic particles the histograms of which were dissimilar and those with dissimilar cell types yielded submicroscopic particles the histograms of which were similar.

Clinical status of Hodgkin's cases in relation to histograms.—The case history of each Hodgkin's patient at the time of biopsy was studied in order to determine whether the clinical history of the

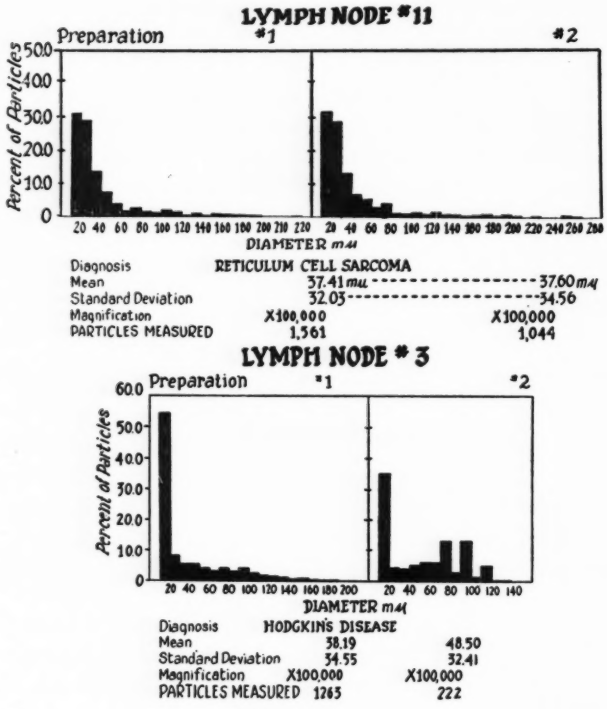


CHART 2

patient was related in any way to the form of the histogram. No correlation could be found between the mean particle size or the percentage of particles smaller than 60 mμ and the duration of the disease at the time of biopsy, the interval between previous treatment and biopsy, the type of treatment prior to biopsy, and the rate of progress of the disease.

DISCUSSION

Although the smallest particle measured in the lymph node high gravity sediments was 10 mμ, it would be expected that smaller particles existed in all samples. In many cases, particles smaller than 10 mμ were seen on the photographic negatives at a magnification of 100,000×; however, owing to the limit of resolution of the microscope and the poor electron absorption ability of these small particles, accurate measurement of their sizes was impossible. Therefore, while the maximum concentration of particles in the lymphoma lymph node cells appears to be in the 10–20 mμ size range, it is possible that the highest concentration of particles in these cells may be smaller than 10 mμ.

The statistical evaluation of the histograms indicates a smaller mean particle size in the lymphoma lymph nodes than in the non-neoplastic lymph nodes. The reason for this difference has not been determined. The possibility that fragmented mitochondria contribute to the population of small particles cannot be overlooked. Dalton *et al* (1) have reported that the mitochondria of mouse tumor cells are more fragile than those of normal hepatic cells. A method for the determination of the amount of mitochondria broken up during disruption of lymph node cells and subsequent centrifugation and for a physical comparison of these fragments with those obtained in the high gravity sediments is being investigated.

Qualitatively, no relation could be found between the type of lymph node cells and the form of the histogram. However, the cells of the neoplastic lymph nodes are, in many cases, larger and contain a greater quantity of cytoplasm than those of the non-neoplastic nodes. These facts have been confirmed both histologically and grossly by the fact that a larger quantity of particulate material is recovered in the high gravity sediments of neoplastic lymph nodes than is recovered in the high gravity sediments of non-neoplastic nodes. It may be possible that the increase in cytoplasmic material in the neoplastic cells contributes to the increase in particles 10–30 mμ in size.

The question concerning the origin of the particles obtained in the high gravity sediments has been answered partially in a previous publication (4). The absence of desoxyribosenucleic acid in the high gravity sediments and the presence of intact nuclei in the low gravity sediments suggest that the particles obtained in the 25,000 *g* sediments are essentially cytoplasmic in origin.

The reproducibility of results employing the technics outlined for the construction of histograms has been shown to be satisfactory if a sufficient number of particles photographed in the electron microscope are measured. Under such conditions, histograms of two samples of the same lymph node centrifuged on separate occasions resemble each other. However, if an insufficient number of particles is measured, histograms of duplicate lymph node samples do not resemble each other.

SUMMARY

The statistical evaluation of histograms constructed from electron micrographs of particles

TABLE 1

Statistical Evaluation of Histograms

Magnification: 100,000

Lymph node no. and diagnosis	Mean particle size (mμ)	No. of particles counted	Standard deviation	Percentage of particles in groups 10–60 mμ 10–20 mμ	20–30 mμ	30–40 mμ	40–50 mμ	50–60 mμ
No. 10 Hodgkin's disease	58	725	50	16	20	12	11	8
9 Hodgkin's disease	54	1,294	53	23	24	14	7	4
5 Hodgkin's disease	44	1,304	64	50	19	8	5	2
8 Hodgkin's disease	35	1,069	37	45	19	9	6	4
3 Hodgkin's disease	36	1,263	36	54	8	6	6	4
6 Hodgkin's disease	35	794	34	48	7	11	8	7
4 Hodgkin's disease	31	875	37	53	22	5	2	4
Av.	42	1,046	44	41	17	9	6	5
11 Reticulum-cell sarcoma	37	1,561	33	32	29	13	8	4
12 Reticulum-cell sarcoma	24	1,286	27	48	38	6	3	1
13 Lymphosarcoma	31	891	27	40	27	15	4	3
14 Lymphatic leukemia	17	1,423	7	88	9	2	0	0
Av.	27	1,290	23	52	26	9	4	2
15 Metastatic medullary carcinoma (breast)	70	1,542	53	14	14	9	12	7
16 Metastatic medullary carcinoma (breast)	49	2,510	39	17	24	17	11	7
Av.	59	2,026	46	15	19	13	12	7
17 Lymphadenitis	51	1,044	45	30	9	13	9	7
21 Reticulum-cell hyperplasia	50	1,118	33	20	17	7	8	19
25 Inflammation	77	1,275	46	8	6	6	7	9
Av.	59	1,146	41	19	10	9	8	12

TABLE 2

Histologic Cell Types in Relation to Distribution of Particle Sizes

Lymph node no.	Histologic diagnosis	Percentage of Histologic Cell Type: Reticulum cells (including Hodgkin's cells)	Lymphocytes	Granulocytes	Clasmatocytes	Fibroblasts	Plasma cells	Endothelial cells	Eosinophils and polymorphonuclear neutrophils	Carcinoma cells	Percentage of particles smaller than 60 mμ	Mean particle size (mμ)
1	Hodgkin's disease	25	72	1	2	<1					81	38
2	Hodgkin's disease	17	74	6			3				92	33
3	Hodgkin's disease	13	79		1	1		<1	6		78	36
4	Hodgkin's disease	40	42				<1		18		86	31
5	Hodgkin's disease	70	30								83	44
6	Hodgkin's disease	63	3	34							81	35
8	Hodgkin's disease	23	73	1		1	<1	<1			83	35
9	Hodgkin's disease	26	62	8	<1	2	<1	1			72	54
10	Hodgkin's disease	73	18	9							68	58
11	Reticulum-cell sarcoma	38	62								85	37
13	Lymphosarcoma	6	92	<1	<1			1			89	31
14	Lymphatic leukemia	3	97								99	17
15	Metastatic medullary carcinoma (breast)					2				98	55	70
16	Metastatic medullary carcinoma (breast)					3				97	76	49
17	Lymphadenitis	3	90		5		<1	1			68	51
19	Chronic lymphadenitis suggestive of infectious mononucleosis	27	66		2	5					24	94
21	Reticulum-cell hyperplasia	29	70		<1		<1				70	50
22	Reticulum-cell hyperplasia	5	65		30						53	69

obtained in the 25,000 *g* centrifugation sediments from Hodgkin's disease, other lymphomas, carcinomas, and non-neoplastic lymph node cells was undertaken. The most striking characteristic of the four general groups of lymph nodes studied was the percentage of particles smaller than 60 mμ. In the Hodgkin's disease group and in the lymphoma group other than Hodgkin's disease, the maximum number of particles occurred in the 10–20 mμ size range; in the carcinomas, the greatest number was in the 20–30 mμ size range. The non-neoplastic preparations as a group had no predominant size range.

The average mean particle size of the Hodgkin's disease group was 42 mμ; that of the lymphomas other than Hodgkin's disease was 24 mμ; and the average mean particle sizes of the carcinoma and non-neoplastic group were identical, 59 mμ.

No correlation could be found between the form of the histogram and the distribution of histologic cell types in the lymph nodes, and no correlation could be found between the form of the histogram and the clinical status or type of treatment of the Hodgkin's patients at time of biopsy.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the assistance of Dr. Ransom W. Whitney, Director of the Statistics Laboratory, Ohio State University, who directed the statistical evaluation of the histograms; of Mr. G. Adolph Ackerman, who performed the differential cell counts of the lymph node sections; and of Mrs. Bette M. Hainer, who assisted in the electron microscope work.

REFERENCES

1. Dalton, A. J.; Kahler, H.; Kelly, M. G.; Lloyd, B. J.; and Striebich, M. J. Some Observations on the Mitochondria of Normal and Neoplastic Cells with the Electron Microscope. J. Nat. Cancer Inst., **9**:439–49, 1949.
2. Evans, A. S., and Melnick, J. L. Electron Microscope Studies of the Vesicle and Spinal Fluids from a Case of Herpes Zoster. Proc. Soc. Exper. Biol. & Med., **71**:283–86, 1949.
3. Hogeboom, G. H.; Schneider, W. C.; and Pallade, G. E. Cytochemical Studies of Mammalian Tissues. I. Isolation of Intact Mitochondria from Rat Liver; Some Biochemical Properties of Mitochondria and Submicroscopic Particulate Material. J. Biol. Chem., **172**:619–35, 1948.
4. Hoster, M. S.; McBee, B. J.; Rolnick, H. A.; Van Winkle, Q.; and Hoster, H. A. Macromolecular Particles Obtained from Human Neoplastic and Non-neoplastic Lymph Nodes. I. Procedure and Preliminary Results. Cancer Research, **10**:530–38, 1950.
5. Porter, K. R., and Thompson, H. P. A Particulate Body Associated with Epithelial Cells Cultured from Mammary Carcinomas of Mice and of a Milk-Factor Strain. J. Exper. Med., **88**:15–24, 1948.
6. Schneider, W. C., and Hogeboom, G. H. Cytochemical Studies of Mammalian Tissues: The Isolation of Cell Components by Differential Centrifugation: *A Review*. Cancer Research, **11**:1–22, 1951.

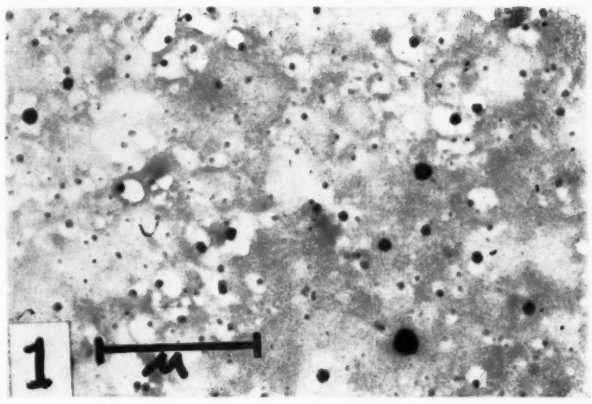


FIG. 1.—Particles in the sediment (25,000 *g*) obtained from a homogenate of Hodgkin's lymph node cells. Mag. ×20,000.

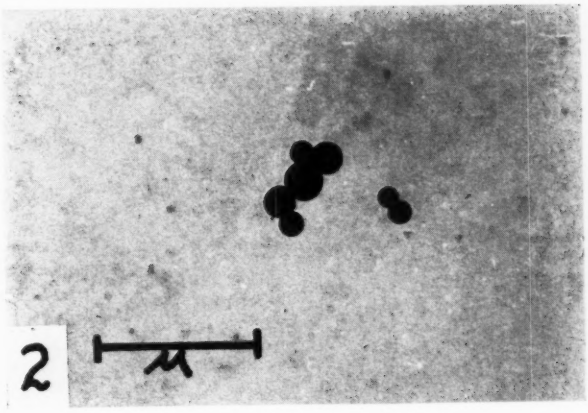


FIG. 2.—Particles in the sediment (25,000 *g*) obtained from a homogenate of giant follicular hyperplastic lymph node cells. Mag. ×20,000.

Announcement

A Summer Course in the Principles and Technics of Tissue Culture

The Tissue Culture Association is again sponsoring a course of lectures and laboratory instruction in the principles and technics of tissue culture. The course will be under the direction of Dr. John H. Hanks, the Leonard Wood Memorial, Harvard Medical School, and will be given in the laboratories of the Mary Imogene Bassett Hospital, Cooperstown, N.Y. It will start on August 6th and end on August 28th, 1952. Tuition will be seventy-five dollars.

The course is designed specifically for postgraduates (M.D. or Ph.D.) who plan to use cultured tissues in their research or teaching. Requests for application forms should be addressed to Dr. Mary S. Parshley, College of Physicians and Surgeons, 630 West 168th Street, New York 32, N.Y., and should be completed and returned to her not later than April 15th. Successful candidates will be notified about May 1st.